DOCTORAL THESIS

The Effect of Exogenous Estrogen and Progesterone Administration on Exercise Tolerance in Active Women

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The Effect of Exogenous Estrogen and Progesterone Administration on Exercise Tolerance in Active Women.

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DECLARATION

This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Doctor of Philosophy. This thesis represents my own original work towards this research degree and contains no material which has been previously submitted for a degree or diploma at this university or any other institution, except where due acknowledgement is made.

Signed: _____

Rhiannon N. Fisher

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NAVIGATION OF THE THESIS

The present thesis sets out to investigate the immunological response to elite surf lifesaving (SLS) competition, in attempt to observe how multidisciplinary SLS athletes respond to ironman format surf races. Following an initial investigation into a single multidisciplinary SLS event, it became evident that further investigation into the gender difference in immune response and endocrinological response to exercise was warranted; in particular the role of ovarian hormones, namely estrogen and progesterone. Despite the growing participation rates in SLS, it is still considered a low participation sport, predominantly due to the demographical and logistical constraints associated with the sport requiring participants to reside and undertake training at or in close proximity to coastal regions. Additionally, environmental temperatures do not always promote water participation in some parts of Australia, and indeed the world. A direct consequence of low participation numbers results in few SLS athletes being available to participate in research, and consequently, participant recruitment becomes difficult. In addition, due to the seasonal nature of ocean-based sports, it became clear a substantial body of work for the present thesis could not be undertaken within an appropriate timeframe. As a result, the focus of the thesis changed to recreationally active women, with the intent to understand fundamental changes in the immunological and endocrinological response to exercise in relation endogenous and exogenous estrogen and progesterone secretion. It is the hope of the researcher that the findings of this thesis in relation to recreationally active women may form the basis of further research into elite female athletes.

Prior to investigating the role of endogenous and exogenous estrogen and progesterone secretion to the immunological and endocrinological response to exercise, it was important to investigate the benefits and barriers for oral contraceptive use or non-use within a physically active population. Following this investigation into the perceived benefits and barriers towards oral contraceptive use and non-use in women of varying physical activity levels, it became evident that we needed to explore exactly how oral contraceptive (OC) use or non-use affects exercise tolerance. In particular the present thesis will focus on immunological and endocrinological challenges of exercising in varying environmental temperatures. The aforementioned parameters of human physiology were identified to warrant investigation as the endocrinological and immunological systems are interrelated, with the manipulation of environmental temperature important due to the geographical location of the recreationally active women investigated

throughout the present thesis. Additionally, exogenous estrogen and progesterone secreted throughout OC administration, have been reported [1] to strongly bind to the endogenous estrogen and progesterone receptors located within the hypothalamic and pituitary regions of the brain, it is likely that OC may influence the hypothalamic response [2] to exercise to a greater extent than the normal menstrual cycle, with core body temperature, the mucosal immune and stress response to exercise vulnerable to changes in the functioning of the hypothalamus.

The present thesis is comprised of nine chapters. Chapter one demonstrates the problem within the population and identifies the gaps in the literature, highlighting the purpose of the research as well as the research questions. Chapter two explores the current literature surrounding this research problem with emphasis placed upon the exercising population and females. Chapter three is experimental study one which explores the validity and reliability of a point of care saliva analysis test which will be used in subsequent studies and poses substantial benefits for applied sport science endocrinological testing. Chapter four is experimental study two which is an exploratory study investigating the immunological response to a single surf life saving event. Chapter five is experimental study three and explores the benefits and barriers for oral contraceptive use or non-use in women of varying physical activity levels. Chapter six is experimental study four and explores the mucosal immune and endocrinological response to incremental exercise in recreationally active women taking oral contraception compared with normally menstruating women. This chapter also explores the core body temperature (T_C) response in women taking oral contraception to incremental exercise in thermo-neutral (TN) conditions (22°C; r.h. 50%) and hot and humid (HH) (35°C; r.h. 50%) environments and how this affects the mucosal immune system and the stress response. Chapter seven provides an overall discussion from all experimental studies and outlines future research to further the results attained from the present thesis. Chapter eight explains the principles behind the testing methodology used throughout the present thesis.

ABSTRACTS

Chapter 3 (Experimental Study 1 – Paper 1)

Saliva analysis is commonly used in applied sport science research and practice due to convenience and ease of sample collection with traditional measures requiring immediate refrigeration and taking several hours to analyse. The aim of the present study was to evaluate the validity and reliability of measuring salivary cortisol concentration ([sCort]) *in situ* with the Individual Profiling (IPRO) oral fluid collector (OFC) method against the Salimetrics oral swab (SOS) and passive drool (PD). Ten (n = 10, male = 5 and female = 5) healthy, recreationally active university students volunteered to participate in the present study. Participants provided three samples in trial one (i.e. one of OFC, SOS and PD). In trial two participants repeated the sample procedure from trial one, with four participants providing duplicate OFC swabs for reliability analysis. The duplicate swabs were analysed on duplicate lateral flow devices (LFD) to test for reliability. No significant difference was found between OFC and SOS ($p = 0.881$) and PD ($p = 0.145$) measures, showing good agreement with no bias. Both duplicate OFC and LFD samples were not significantly different from another, with an ICC of 0.890 and 0.850 respectively. The present study demonstrates the IPRO method to be a valid and reliable measure of [sCort] in recreationally active individuals, indicating a useful and convenient measure for salivary cortisol testing in field environments.

Chapter 4 (Experimental Study 2 – Paper 2)

The present study examined the response of salivary immunoglobulin A (s-IgA) to endurance SLS competition. Elite SLS men (n = 10) and women (n = 8) volunteered to participate in the present study. Saliva samples of 0.5 mL were collected daily commencing 54 hr pre-event to 61 hr post-event. Saliva samples were assessed using a portable system. Saliva samples were collected at rest and prior to physical exertion or activity at approximately 0500 hours to determine the pre-and post-competition baseline values, while the post-event sample collection occurred within 30 min of race completion for each athlete, between approximately 1300 – 1600 hours.

A significant ($p < 0.05$) increase in s-IgA concentration ([s-IgA]) was observed 61 hr post-event (49.21 ±8.8 µl/mL) in males compared to all other time-point samples. The results of the present study indicate that there is a non-significant increase in [s-IgA] for both genders immediately

following an endurance SLS event, lasting approximately 90 min. The mechanism responsible for the non-significant increases in [s-IgA] observed post-event in both genders and significant increases in [s-IgA] observed 61 h post event in males remains unclear and may reflect an immune-compensatory effect to protect the athlete from the acquisition of infection. Surf life saving athletes and coaches should monitor [s-IgA] throughout major competition periods to observe fluctuations in mucosal immunity, which may reflect a predisposition to infection, and tailor exercise and recovery prescription accordingly to minimise the risk of infection and subsequent declines in performance.

Abstract Insert

Upon reflecting on this paper and the possible role of gender in the mucosal immune response to multidisciplinary surf life saving competition, it became apparent that the menstrual cycle and OC use may have implications for our results, which raised several research questions, ultimately changing the direction of the present thesis. Do ovarian hormones influence the acute mucosal immune and stress response to intense exercise? In particular do exogenous ovarian hormones in the form of oral contraceptives cause different effects than endogenous secretions throughout the normal menstrual cycle? In addition, do OC affect other parameters of physiology, including the stress response to intense exercise and the ability of the active female to regulate T_C when exercising? In an attempt to answer these questions, several studies were designed; a qualitative survey-based analysis of OC use in women of varying physical activity levels to identify if exercise plays a role in OC use or non-use in Australian women, as well as the perceived benefits and barriers towards OC use within these women. Further studies were conducted to investigate the effect of exogenous estrogen and progesterone in the form of OC on exercise tolerance in recreationally active women, with a focus on T_C, s-IgA and salivary cortisol (sCort).

Chapter 5 (Experimental Study 3 – Paper 3)

The present paper explores the role of physical activity levels on OC use in active, athletic Australian women. The benefits and barriers for OC use are also explored. A total of 125 Australian women (29.4 ±8.1 yr) completed a survey on their menstrual cycle and OC use. Women were categorised based on weekly exercise levels and intensities as either untrained (n = 26; 32.2 ±7.3 yr: UT), recreationally active (n = 44; 30.6 ±6.8 yr: REC) or trained (n = 55; 27.1 ±8.3 yr: TR). No significant differences ($p > 0.05$) were observed for OC use between TR (47%), REC (39%) and UT (31%) women. Birth control (90%), cycle control (86%) and a reduction in

menstrual disturbances (88%) were the major benefits of OC use reported by all women, whereas the major barriers for OC use showed more variation between women. Physical activity levels do not appear to play a role in OC use in Australian women, although may indirectly affect the perceived needs of a woman.

Chapter 6 (Experimental Study 4 – Paper 4)

A key component of exercise tolerance is attenuating changes in T_C throughout exercise, particularly in HH conditions. Elevations in T_C may place the cardiac output demands of the exercising individual under more stress to meet metabolic and thermoregulatory loads, subsequently increasing the stress and immune response to exercise. Endogenous progesterone has been associated with increases in T_C throughout the luteal phase of the menstrual cycle. Considering exogenous progesterone in monophasic OC is secreted in higher amounts, it is possible that taking OC may augment rises in T_C in women. The aim of the present study was to investigate the role of exogenous progesterone and estrogen administration on T_C at rest and throughout exercise. Sixteen (n = 16) recreationally active women (8 = normally menstruating; womenNM, 8 = taking OC; womenOC) aged 22.1 ±3.2 yr completed two incremental cycling tests (52.5 min) during the early follicular phase for womenNM and during the active pill phase for womenOC. One was conducted in TN conditions (22°C; r.h. 50%; TN) and one in hot and humid conditions (35°C; r.h. 50%; HH). Salivary cortisol ([sCort]), salivary Immunoglobulin A ([sIgA]) were collected at baseline (approximately 0500 hours), following 30 min of acclimation (approximately 0530 hours) and immediately following exercise (approximately 0800 hours) in both TN and HH trials. No significant difference in [sCort] or [sIgA] was observed between groups. WomenOC demonstrated significantly ($p < 0.05$) higher resting T_c compared with womenNM in both conditions, and T_C remained significantly ($p < 0.05$) in womenOC than in womenNM up to 22.5 min into the exercise test.

LIST OF PUBLICATIONS

1. **Fisher, R. N.,** McLellan, C. P., & Sinclair, W. H. (2015). The Validity and Reliability for a Salivary Cortisol Point of Care Test. Journal of Athletic Enhancement, 4:4.

2. **Fisher, R. N.,** Sinclair, W. H., Lovell, D. I., & McLellan, C. P. (2015). The Immunological Response to Surf Life Saving Competition. Journal of Australian Strength and Conditioning. 23(2), 15-20.

3. **Fisher, R. N.,** Sinclair, W. H., McLellan, C. P., & Minahan, C. L. (2015). Prevalence and the associated Benefits and Barriers of Oral Contraceptive use in Australian women grouped for physical activity level. *Currently under peer-review.*

4. **Fisher, R. N.,** McLellan, C. P., Sinclair, W. H., & Minahan, C. L. (2015). Salivary cortisol and IgA response to incremental cycling in a thermally-stressful environment; the role of oral contraceptives and core body temperature. *Currently under peer-review.*

LIST OF CONFERENCE PROCEEDINGS

Fisher, R. N., Sinclair, W. H., McLellan, C. P., & Minahan, C. L. The role of physical activity in oral contraceptive use in Australian women. Symposium: Oral contraception and the menstrual cycle in exercise science and sports medicine research – should it be considered? Sports Medicine Australia Conference, October 2015, Sanctuary Cove (Oral Presentation & Abstract).

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LIST OF SYMBOLS AND ABBREVIATIONS

ACTH – Adrenocorticotropic Hormone

AEE – Apical Early Endosomes

ANOVA – Analysis of Variance

ARE – Apical Recycling Endosomes

BLa – Blood Lactate

BMD – Bone Mineral Density

BUHREC – Bond University Human Research Ethics Committee

CAR – Cortisol Awakening Response

CBG – Corticosteroid Binding Globulin

CHO - Carbohydrate

CI – Confidence Interval

CNS – Central Nervous System

COC – Combined Oral Contraceptive

CRH – Corticotropin Releasing Hormone

CV – Coefficient of Variation

CVD – Cardiovascular Disease

DALT – Duct-Associated Lymphoid Tissue

DEXA – Duel Energy X-ray Absorptiometry

DVT – Deep Vein Thrombosis

EDTA – Ethylenediaminetetraacetic Acid

EIA – Enzyme Immunoassay

ELISA – Enzyme Linked Immunosorbent Assay

ERα - Estrogen Receptor Alpha

ERβ – Estrogen Receptor Beta

ESSA – Exercise and Sport Science Australia

Fab – Fragment Antibody Binding

Fc – Fragment Crystallised

FSH – Follicle Stimulating Hormone

GnRH – Gonadotropin Releasing Hormone

H_2O – Water

HH – Hot and Humid

HPA Axis – Hypothalamic Pituitary Adrenal Axis

HR – Heart Rate

ICC – Intraclass Correlation

ICS- Immunochromatographic Strip

IPRO – Individual Profiling

J Chain – Joining Chain

LFD – Lateral Flow Device

LH – Luteinizing Hormone

LT1 – Lactate Threshold 1

MALT – Mucosa-Associated Lymphoid Tissue

mRNA – Messenger Ribonucleic Acid

NE – Norepinephrine

NK-$_K$B - Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

NSB – Non-Specific Binding

OC – Oral Contraception

OFC – Oral Fluid Collector

PARQ – Physical Activity Readiness Questionnaire

PCOS – Polycystic Ovarian Syndrome

PD – Passive Drool

pIgA – Polymeric Immunoglobulin A

pIgR – Poly-Immunoglobulin Receptor

PMS – Premenstrual Syndrome

PO/AH – Preoptic / Anterior Hypothalamus

PR – Progesterone Receptor

$\dot{Q}$ – Cardiac Output

r.h. – Relative Humidity

REC – Recreationally Active

RER – Resting Exchange Ratio

RPE – Rating of Perceived Exertion

SC – Secretory Component

SCA – Saliva Collection Aid

SCN – Suprachiasmatic Nucleus

sCort – Salivary Cortisol

SD – Standard Deviation

SEM – Standard Error of the Mean

s-IgA – Salivary Immunoglobulin A

SLS – Surf Life Saving

SNS – Sympathetic Nervous System

SOS – Saliva Oral Swab

T1 – Trial One

T2 – Trial Two

T_C – Core Body Temperature

T_{CTHLD} – Core Body Temperature Threshold

TH1 – T-Helper 1

TH2 – T-Helper 2

TMB – Tetramethylbenzidine

TN – Thermo-neutral

TR – Trained

URTI – Upper Respiratory Tract Infection

UT – Untrained

VE – Minute Ventilation

$\dot{V}O_2$ Max – Maximal Oxygen Consumption

$\dot{V}O_2$ Peak – Peak Oxygen Consumption

WomenNM – Women Considered to be Normally Menstruating

WomenOC – Women Taking Oral Contraception

X^2 – Chi Square

3G – Third Generation

Units of Measurement:

°C – Degrees celsius

% - Percentage

ml - Millitres

± - Plus or minus

µg/mL – Microgram per millilitre

L/min – Litres per minute

$ml/kg^{-1}/min^{-1}$ – Millilitres per kilogram per minute

$ml/kg^{-1}LBM/min^{-1}$ – Millilitres per kilogram of lean body mass per minute

min – Minute

hr – Hour

yr – Year

mo – Month

wk - Week

mg/d – Milligrams per day

> - Greater than

< - Less than

≥ - Equal to or greater than

MW – Molecular weight

kDa – Kilodalton

Da – Dalton

METS – Metabolic equivalents

α - Alpha

B – Beta

bpm – Beats per minute

g/L – Grams per litre

ml/min – Millilitres per minute

µL – Microliter

~ - Approximately

g – Grams

kg – Kilograms

mmol/L – Millimoles per litre

w - Watts

μSv – Microsieverts

mm - Millimetres

g (force) – Forces exerted by gravity

nmol/L – Nanamoles per litre

rpm – Revolutions per minute

cm – Centimetres

g/cm^2 – Grams per square centimetre

nm – Nanometre

m – Metres

pg/ml – Picogram per millilitre

ng/ml – Nanogram per millilitre

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CHAPTER ONE

1. 1 Introduction

Males and females exhibit fundamental differences in physiological and morphological parameters [3] which may restrict aspects of exercise performance. Maximal oxygen uptake ($\dot{V}O_2$ max) is considered the gold standard measurement of cardiorespiratory endurance [4]. In general, the average absolute $\dot{V}O_2$ max (L/min) in males is approximately 50% greater than in females [5], and following correction for body mass ($ml.kg^{-1}.min^{-1}$) or lean body mass ($ml.kgLBM^{-1}.min^{-1}$) the difference between males and females is reduced to 20-30% and less than 15% respectively [6], indicating distinct gender differences. If, when all variables are equal, women consistently exhibit lower $\dot{V}O_2$ max than males, then a rationalisation of the mechanism or mechanisms associated with that difference is a meaningful pursuit to optimise cardiorespiratory performance in female athletes and active women. One possible explanation could be the influence of the menstrual cycle, specifically the presence of estrogen and progesterone. Endogenous and exogenous estrogen and progesterone appear to affect $\dot{V}O_2$ max performance with women exposed to exogenous hormones [7, 8], in the form of OC exhibiting lower $\dot{V}O_2$ max compared with women who are considered normally menstruating i.e. not taking any form of hormonal contraception. It remains unknown if this decrease in aerobic capacity in response to exogenous estrogen and progesterone secretion is due to the structure of the molecule and the subsequent effect on receptor binding, or due to the shear amount of synthetic hormone exposure. Even though the mechanism behind this relationship remains equivocal, it does substantiate claims that ovarian hormones contribute to the gender difference in exercise performance. Considering the potential relationship between ovarian hormones and exercise performance, it is likely that other parameters of physiology may be affected in relation to the secretion of estrogen and progesterone.

Women have been reported to exhibit higher T_C than males at rest [9] and in response to exercise [10], and even though this difference is lowered when body surface-to-mass ratios, adipose tissue percentage and aerobic capabilities are controlled for [11], women still appear to have an altered thermoregulatory response compared with males. In particular females appear to have lower sweat rates than males in response to exercising in hot conditions [11]. The menstrual cycle and subsequent fluctuations in estrogen and progesterone may contribute to the higher T_C previously observed [9, 10] in females compared with males. Progesterone in particular has been shown [12-15] to increase the core temperature threshold ($T_{C\ THLD}$) for cutaneous vasodilation and

subsequently sweating, consequently also leading to an elevated T_C. Interestingly, little is known about the effect of exogenous progesterone on T_c and the $T_{C\ THLD}$ for cutaneous vasodilation. Considering a greater amount of progesterone is secreted during OC use compared with the normal menstrual cycle, it is likely that the progesterone-induced changes to T_C could be amplified in women exposed to exogenous progesterone. If women taking OC experience higher (progesterone-induced) T_C and $T_{C\ THLD}$ for cutaneous vasodilation, it is plausible they would experience a greater stress response to exercise, ultimately compromising their ability to sustain the metabolic demands of exercise.

Cortisol is a corticosteroid, commonly secreted when the body is experiencing stress i.e. intense exercise, and can affect the secretion of gonadotropin releasing hormone (GnRH) [16, 17], which is involved in regulating the menstrual cycle [18]. Throughout the normal menstrual cycle fluctuations in cortisol have been reported, including decreases in the follicular phase and increases throughout the ovulatory phase [19, 20]. It is possible that this fluctuation in cortisol concentrations could be related to changes in T_C, as the observed increases in cortisol secretion occur in the ovulatory phase when progesterone is starting to rise, subsequently causing the surge in T_C. The lower cortisol concetrations reported in the follicular phase of the menstrual cycle further demonstrate an association between T_C and cortisol secretion, with estrogen the predominant hormone secreted, which is known to lower T_C [14, 15]. In addition, women taking OC appear to experience higher resting levels of serum cortisol compared to normally menstruating women [21]. It is therefore likely that not only would women taking OC experience a higher T_C due to greater progesterone secretion, it is possible that they would also experience a greater stress reponse to exercise, which may affect other parameters of health and performance.

Chronic elevations in resting cortisol concentrations may affect mucosal immune system functioning [22, 23], through the down regulation of B-Lymphocyte mobilisation [24], specifically [s-IgA]. Salivary Immunoglobulin A is the major antibody present in mucosal fluids and its function is to inhibit viral and bacterial replication and attachment to the mucosal epithelium of the upper respiratory tract, thereby preventing pathogen entry into the body [25-28]. The suppression of [s-IgA] is closely associated with the anecdotal increase in respiratory tract infections (URTI) in athletes [29-31], particularly surrounding periods of high stress or load i.e. intense training. Initially the present thesis will focus on surf lifesaving (SLS) athletes, and the effect of competition on [s-IgA]. Surf lifesaving is a multidisciplinary ocean-based sport,

involving substantial physical conditioning and skill training to achieve 'elite' success. The specific group of SLS athletes investigated in the present thesis were national level athletes, competing in the Kellogg's Nutri-grain Ironman and Telstra Ironwoman Series' at the time of testing. The Kellogg's Nutri-grain Ironman and Telstra Ironwoman Series' are exclusive, elite competitions which involve up to six separate races, with the overall series winner named champion Irornman or Ironwoman. Each of the six races are a different length, format i.e. sprint vs. endurance, and are held at various beaches across Australia. All races encompass the fundamental Ironman or Ironwoman skills of soft-sand running, ocean swimming, Malibu board paddling and surf ski paddling. The multi-event nature of surf lifesaving competitions may result in many athletes failing to fully recover between events and competitions and subsequently experiencing declines in performance, as anecdotally reported by SLS coaches and athletes. Therefore initially the present thesis sets out to investigate the [s-IgA] response to elite SLS competition. The association between [s-IgA], stress and overall immune health are the predominant reasons why the s-IgA response is often monitored in applied sport science research. However, due to the impracticality of conducting research with a select group of elite athletes, as well as the expenses associated with travel to attend various surf life saving races, the focus of the present thesis alters to recreationally active women. Importantly, lymphocytes exhibit receptors for ovarian hormones [32] and can be affected by the menstrual cycle and OC administration. Estrogen in particular is associated with enhanced immune function [33], with [s-IgA] reported to rise sequentially with estrogen levels throughout the menstrual cycle [34]. However, considering the total concentration of exogenous estrogen secreted in OC is lower than throughout the normal menstrual cycle, it is possible that women taking the OC may exhibit less s-IgA availability on the mucosal surfaces of the upper respiratory tract, potentially predisposing them to infection.

Traditional measures of s-IgA and salivary cortisol (s-Cort) have involved expensive and often time-consuming laboratory tests. These laboratory tests, such as the gold-standard enzyme linked immune-sorbent assay (ELISA) method can be impractical for applied exercise and sport science research, due to strict storage and preparatory guidelines. The ELISA method can also take several hours to complete the sample analysis. The present thesis also investigates the reliability and validity of a novel point of care s-Cort method against the gold-standard ELISA method.

1. 2 Significance of the Research

Gender differences exist in relation to exercise performance which may, in part, be attributable to ovarian hormones estrogen and progesterone. However, evidence is emerging that indicates endogenous and exogenous forms of these hormones may elicit varying effects on the health and exercise performance of active females, and warrants individual investigation. In particular the interaction between progesterone secretion and increases in T_C and subsequent thermoregulatory demands is thought-provoking and could have major implications for women exercising in hot climates such as northern Australia. The additional stress placed on women exercising in hot conditions when exposed to high levels of progesterone could induce greater cortisol secretion which may affect several parameters of health and performance. In particular s-IgA suppression is associated with rising cortisol levels, and is also known to respond to ovarian hormone secretion throughout the menstrual cycle. It is important to investigate how [sCort] and [s-IgA] respond at rest and in response to exercise in relation to endogenous and exogenous ovarian hormone secretion as this may have consequences for the health and performance of the exercising woman.

Currently, little is known about the T_C, stress and mucosal immune response to acute exercise in HH conditions in normally menstruating women compared to women taking OC. This investigation has the potential to provide a unique insight into the way OC may be affecting health and performance in women who often undertake exercise in warmer climates. Additionally, there is little qualitative understanding of the relationship between physical activity levels and OC use or non-use in women and the perceived benefits and the barriers for OC use in Australian women remains largely unexplored, despite an increasing prevalence of women taking OC in Australia and worldwide [35]. The lack of understanding of how taking OC may affect exercise performance and the increasing usage rates of OC in active women and female athletes highlights the need to explore the effect of exogenous estrogen and progesterone secretions on exercise tolerance, as well as the perceived benefits and barriers towards OC use.

1. 2 Research Questions

The present thesis will investigate several questions associated with the effect of exogenous estrogen and progesterone administration on several parameters of exercise tolerance in recreationally active women, as well as the perceived benefits and barriers towards taking OC in Australian women.

1. Is the Individual Profiling (IPRO) point of care device a valid and reliable method of monitoring salivary cortisol concentration?

2. What is the Salivary Immunoglobulin A response to a single, multidisciplinary surf lifesaving event?
3. Do male and female multidisciplinary, surf lifesaving athletes exhibit a different immune response to competition?
4. What is the prevalence of OC use or non-use in Australian women? Is there an association between physical activity levels and OC use or non-use in these women?
5. What are the perceived benefits and barriers towards oral contraceptive use (or non-use) in Australian women?
6. What is the effect of OC use on the mucosal immune and endocrinological response to incremental exercise?
7. Does OC affect the T_C response to incremental exercise in thermo-neutral and hot and humid environments?
8. Does exercise in a hot and humid environment alter the mucosal immune and endocrinological response in women taking OC?

1. 3 Research Progress Linking the Experimental Studies

The present thesis will investigate several questions associated with the effect of exogenous estrogen and progesterone administration on several parameters of exercise tolerance in recreationally active women, as well as the perceived benefits and barriers towards taking OC in Australian women. To achieve this aim, four experimental research studies were undertaken and are presented as chapters three, four, five and six of the thesis.

Each experimental study within the present thesis was designed to build upon the preceding chapter to enhance our understanding of the influence of exogenous estrogen and progesterone administration on exercise tolerance in active women.

1. 3. 1 Experimental Study One

In preparation for experimental study four of the present thesis which involved saliva analysis to monitor cortisol concentrations, a validity and reliability study for a new point of care saliva analysis test was completed. The IPRO device is a novel method of saliva analysis allowing sport scientists to collect and monitor salivary analytes in a field setting. The purpose of experimental study one was to establish the validity and reliability of the IPRO device for monitoring salivary cortisol concentrations against the gold standard

Enzyme-Linked Immunosorbent Assay (ELISA) method to ensure the accuracy of this method for use throughout the present thesis.

1. 3. 2 Experimental Study Two

Experimental study two examined the immunological response to a single endurance (90 min) SLS ironman event, which involved monitoring [s-IgA] across a six day period surrounding the SLS event, including two measures on as many days prior to and three measures in as many days following the event collected at approximately 24 hr intervals, with two additional samples on the day of the race, collected at approximately 5 hr intervals. Experimental study two was conducted to gain a novel insight into the acute mucosal immune (s-IgA) response surrounding a single SLS ironman and ironwoman event. This particular group of athletes were chosen due to the intense nature of training and preparation which is required to compete at the elite level and because no data currently existed within this population regarding mucosal immune health. Due to the absence of information regarding OC use or menstrual cyclicity, several additional research questions were developed and evolved and changed the focus of the present thesis towards females only, and the effect of varying levels of estrogen and progesterone, as well as the effect of exogenous secretions in the form of OC. However, due to geographical constraints and athlete availability following experimental study two, it became clear further research into the acute mucosal response to exercise would be problematic within this sample population. Therefore, the decision was made to alter the focus of the population from elite SLS athletes to recreationally active women, as this would allow recruitment and the conduction of the remaining studies, which focus on females, to occur more efficiently.

1. 3. 3 Experimental Study Three

Following on from the previous investigation into the immunological response to elite SLS competition, and the subsequent questions raised in regards to OC use in women of varying physical activity levels, experimental study three aimed to gain a greater insight into the prevalence of, and motives for OC use in Australian women of varying physical activity levels ranging from sedentary to athletes. Participants completed a survey identifying their motives for OC use or their reasons for not taking OC. Information regarding the type, frequency and intensities of weekly physical activity was also

gathered in these women to identify any trends in physical activity levels and OC use (or non-use). The information gathered from this investigation provided insight into the prevalence of OC use, the role of physical activity levels in OC use or non-use, as well as the common benefits and barriers towards taking OC in Australian women.

1. 3. 4 Experimental Study Four

Experimental study four examined the mucosal immune and stress response to acute exercise in women. Both s-IgA and cortisol have been shown to be affected by the menstrual cycle, and it is possible that exogenous ovarian hormone exposure may augment these changes, placing women taking OC under more stress during exercise and potentially exacerbating exercise-induced decreases in [s-IgA] subsequently increasing the risk of URTI. Ovarian hormones, in particular progesterone have been associated with rises in T_C. Considering fluctuations in T_C have been shown to occur across the normal menstrual cycle in relation to progesterone secretion, it was intriguing to investigate if exogenous progesterone caused similar affects in women taking OC and if these effects varied between TN and HH environments.

Independently and collectively, each of the four experimental studies presented in the present thesis represent a more robust investigation of effect of exogenous estrogen and progesterone secretion on exercise tolerance in active women than reported previously and contribute to the current understanding of the demands of acute exercise in TN and HH environments in women taking OC.

CHAPTER TWO

Review of the Literature

2. 1 THE MENSTRUAL CYCLE AND EXERCISE TOLERANCE

2. 1. 1 The Menstrual Cycle

On average, the menstrual cycle is 28 days in duration but reportedly [36] ranges between 20 to 45 days in some women [37]. The menstrual cycle can be broken down into three distinct phases, namely the follicular phase, luteal phase and the ovulatory phase [37-39]. The follicular phase begins on day one with menses and culminates with ovulation, while the luteal phase is initiated following ovulation through to the start of the menses [37]. In healthy women aged 19-42 yr, ultrasound studies have reported the follicular phase to last for 14.6 days and the luteal phase to last for 13.6 days in duration [40], however the phases have been reported [41, 42] to last between 10-23 and 7-19 days in the follicular and luteal phases respectively. The large variations in phase length can make it difficult to predict each stage and highlights the uniqueness of the menstrual cycle. Throughout the follicular phase, follicle stimulating hormone (FSH) initiates the production of 15-20 follicles each cycle [43]. Within a normal menstrual cycle one follicle will become dominant, with the remaining follicles undergoing atresia [43]. The dominant follicle will continue to develop by acquiring increasing amounts of FSH, while releasing estradiol [43]. Estrogen levels will continue to rise until the end of the follicular phase at which point a positive feedback mechanism stimulates the secretion of luteinizing hormone (LH) which initiates ovulation [38, 43, 44] at which point the immature ovum is released [39]. Once the ovum is released the granulosa and theca cells, as well as some surrounding tissues join to form the corpus luteum [37, 43] as a means of stabilising the endometrium for implantation of the fertilised egg, which is achieved through the secretion of estrogen and progestin [37]. If fertilisation does not occur, both estrogen and progestin will rapidly decrease, which results in the shedding of the uteral lining as menstrual blood flow [38], at which point the process begins again [37].

2. 1. 2 Hormonal Stimulation of the Menstrual Cycle

The regulation of the menstrual cycle is controlled by a series of hormonal secretions, with the pituitary gland, which stimulates the ovaries to release varying levels of hormones depending on the phase of the menstrual cycle. The regulation of the pituitary gland is controlled by the hypothalamus which responds to internal and external stressors, including exercise and

temperature [45]. Throughout menstruation hypothalamic GnRH and FSH are responsible for stimulating the gonads for regular hormone release [46]. The hypothalamus will release GnRH in a pulsatile manner once per 90 min during the follicular phase and once per 60-70 min in the luteal phase of the menstrual cycle [43] and in response to the secretion of norepinephrine (NE), serotonin, and corticotropin-releasing hormone (CRH) [36]. The release of GnRH stimulates the pituitary gland to secrete LH and FSH [18, 47]. The secretion of FSH is crucial throughout the first week of the follicular phase [43] due to its role in releasing the immature ovum [39]. Luteinizing hormone is necessary for the growth of pre-ovulatory follicles as well as luteinisation and ovulation of the dominant follicle [43]. The levels of LH, FSH and estradiol fluctuate throughout the menstrual cycle , upon peaking in the ovulation phase, hormone levels will decrease into the luteal phase [46]. The release of LH and FSH initiates the secretion of estrogen and progestin from the ovaries [36, 39], by activating aromatase and p450 enzymes [43]. Once released, estrogen and progestin signal the pituitary to control the release of LH and FSH and subsequently the release of GnRH from the hypothalamus, which completes the negative feedback loop [36, 39, 43]; this entire process is under regulation of the hypothalamic-pituitary-ovarian axis [16]. While NE is considered a stimulant of GnRH [36], corticosteroids and CRH reportedly inhibit the release of GnRH [16, 17]. Any disturbance to the hypothalamus, which causes changes in homeostasis such as increased stress associated with high intensity exercise could therefore lead to alterations the hormonal regulation and release from the ovaries, ultimately leading to an altered or absent menstrual cycle, which may have implications for exercising women.

2. 1. 3 Ovarian Hormones

There are two major groups of ovarian hormones which are secreted throughout the menstrual cycle, namely the estrogens and progestins, each of which play a crucial role in menstrual cycle regulation. Estrogens are a group of 18-carbon steroids which promote fat deposition in females, generally surrounding the buttocks, breasts and thighs [48]. Both estrogen and progestin have several forms, the major and most potent form of estrogen is estradiol, with estrone and estriol being less potent , while progesterone is the predominate form of progestin [37]. Progesterone is produced from 19-nortestosterone, 17-OH progesterone derivatives and 19-norprogesterone and is expressed in many forms with different affinities and potencies [49]. Two common types of progesterones are levonorgestrel and norethindrone which bind to progesterone receptors, these progesterones are secreted in high amounts from the corpus leutem following ovulation and remain high until menstruation [50]. Estrogen in its endogenous form is produced from the ovaries

as 17β-estradiol, which binds to both estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) with equal affinity [51, 52]. Upon estrogen activation of ERα or ERβ, two known pathways occur, namely the genomic and non-genomic pathways [53]. In the genomic pathway estrogen passes through the lipid membrane and binds to receptors on the nucleus, which will activate or suppress gene transcription [53]; these actions are generally delayed in onset and prolonged in duration [54]. Whereas, the non-genomic pathway involves the activation of cell membrane receptors causing intracellular messengers including nitric oxide, calcium and kinase to be released [53], which has a rapid onset and is short in duration [54]. Estradiol and progesterone are secreted in varying amounts throughout each phase of the menstrual cycle; estradiol levels are lower during the follicular phase, peaking just before ovulation and decreasing throughout the luteal phase [19, 55], while progesterone levels are at their highest in the luteal phase [46, 47] generally peaking six days following ovulation and progressively declining to pre-ovulatory levels at the beginning of menses [56]. For the purposes of the present thesis, the generic terms *estrogen* and *progesterone* will be used to describe each group of ovarian hormones, unless a specific form of either hormone is stated.

2. 1. 4 Ovarian Hormones and Health

Each ovarian hormone has a different effect on immune system functioning with progesterone reported to inhibit the immune response [57-60], while estrogen was observed [57-59, 61] to stimulate immune functioning in low doses and inhibit the immune response in high doses. It is possible that the association between estrogen and cortisol may contribute to the inhibition of immune functioning throughout periods of high estrogen secretion i.e. the follicular phase, as estrogen has been suggested as a modulator of cortisol [21], with reports [19, 20, 62] of spikes in cortisol during ovulation; immediately following a surge in estrogen secretion. In addition to changes in immune health and stress in relation to fluctuating estrogen and progesterone levels, cardiovascular health can also be affected. Estrogen receptor alpha has several known effects on the cardiovascular system, including initiating an acute vasodilatory response through the release of nitric oxide synthase [63, 64] in the non-genomic pathway, as well as increasing endothelial cell growth and reducing the proliferation of smooth muscle cells in the genomic pathway [53]. Due to the varying secretions of estrogen throughout the normal menstrual cycle, it is possible that the cardiovascular changes [53, 63, 64] which occur in response to ERα activation may vary from phase to phase, subsequently having consequences for the health of women.

2. 1. 5 The Menstrual Cycle and Athletic Performance

While it is possible for estrogen to affect the cardiovascular health of a woman, it is generally accepted that the menstrual cycle does not adversely affect exercise performance in the athletic or general population [65-67]. Maximum oxygen uptake, which is the product of the extraction of oxygen in the tissues and the amount of blood delivered to them [48], has been shown to be unaffected by normal [68-71] or hot conditions [72-74] in relation to the menstrual cycle. Additionally, several measures of athletic performance including aerobic capacity, anaerobic capacity and high intensity endurance ability are not significantly affected by menstrual cycle phase on several physiological tests including maximum HR, maximum minute ventilation (VE), maximum respiratory exchange ratio (RER), and endurance time to fatigue [65]. Moreover, metabolic and cardiovascular responses in untrained and trained females were monitored at rest and during exercise throughout the luteal and follicular phase as well as during menstruation, finding no response was significantly affected by any phase of the menstrual cycle [75], further demonstrating a lack of a relationship between endogenous estrogen and progesterone and exercise performance.

2. 1. 6 Exercise and Common Menstrual Disturbances.

Female athletes and active women often experience changes to their menstrual cycle depending on their level of physical activity [76-80], which may increase the prescription of OC to regulate or simulate a normal menstrual cycle. Menstrual disturbances appear to be common in adolescent female athletes, with reports [78, 81-83] of up to 54% of highly active women experiencing irregularities in their cycles. The most frequently occurring disturbances in athletes include changes to the duration of the menstrual cycle, delayed menarche in young women, as well as oligomenorrhea, dysmenorrhoea and in severe cases secondary amenorrhea [84-87]. It has been reported that up to 90% of women experience symptoms of dysmenorrhoea, including excessive menstrual pain that limits normal activities or requires medication [87-90]. The pain which is characteristic of dysmenorrhoea has been associated with the release of prostaglandins which are secreted in response to increases in myometrial activity [91]. It is also common for women to experience premenstrual syndrome (PMS) which may include breast tenderness, bloating and acne slightly prior to menstruation [92], with these factors potentially limiting or affecting a woman’s desire to engage in exercise.

In severe cases, highly active women may experience secondary amenorrhea, which is characterised by hypothalamic dysfunction leading to complete follicular and luteal suppression, resulting in a lack of estrogen secretion [93, 94]. A lack of estrogen secretion may affect the health and wellbeing of the woman, as estrogen is known to induce positive effects on immune and cardiovascular health [63, 64]. Secondary amenorrhea is more common in females athletes who exhibit smaller physiques with low body fat [95, 96] including endurance athletes such as runners and SLS athletes [97]. Secondary amenorrhea reportedly affects between one and 50% of highly active women but only 2-5% of the general population, suggesting a link between exercise levels and estrogen suppression [97-102]. Lower levels of estrogen were reported in exercising women compared with eumenorrheic women [93, 94], further demonstrating the relationship between exercise and estrogen suppression. It is likely that this absence of ovarian hormones secretion is due to a suppression of GnRH in response to chronically elevated cortisol and CRH secretion following long-term, high intensity exercise. Exercise-induced estrogen suppression may have implications for the health of female athletes and highly active women, with estrogen shown to contribute to decreases in calcium absorption in the bone, affecting development [103, 104]. Estrogen suppression can also negatively affect vascular function, vascular tone, homeostasis, fibrinolysis [105, 106] and white blood cell trafficking, and contribute to a suppression of the mucosal immune system [107].

2. 2 ORAL CONTRACEPTION AND EXERCISE TOLERANCE

2. 2. 1 Oral Contraception

Modern OC contain varying combinations and dosages of synthetic hormones, namely estrogen, and progesterone, depending on the type and brand of OC utilised [108]. Oral contraceptives are used to inhibit the HPA axis from stimulating the secretion of endogenous estrogen and progesterone [109]. The suppression of the HPA axis precludes the production of gonadotropins mid-cycle, which prevents ovulation and consequently, the ability for fertilisation to take place resulting in pregnancy [109, 110]. There are two types of OC; namely combined OC (COC) and progesterone only or mini pills. Progesterone-only mini pills are often utilised when the woman cannot tolerate estrogen [111, 112] and are a less commonly used form of contraception due to statistically preventing approximately 1 in 2 pregnancies [111]. Combined OC are characterised by having a specific dosage of estrogen and progesterone [111]. There are three forms of COC, namely monophasic, biphasic and triphasic, each of which secrete constant, two-phase or three-phase increases in dosages of exogenous ovarian hormones [111, 112] throughout the 21-day

active pill phase respectively. Biphasic and triphasic COC attempt to replicate the normal fluctuations in hormones which occur throughout each stage of the menstrual cycle, while monophasic OC releases the same dosage of estrogen and progesterone throughout the entire cycle [37, 43]. In biphasic OC the dosage of progesterone is altered between days 7-10 and 11-14, while estrogen secretion remains constant [112, 113]. In triphasic OC the dosage of estrogen is increased three times throughout the cycle. The average concentration of estrogen present in monophasic OC pills is 0.03 mg/d, with a range of 0.02 to 0.05 mg/d depending on the brand of OC utilised, while in triphasic OC pills estrogen concentration is predominantly between 0.03 and 0.04 mg/d [114]. The concentration of progesterone in both monophasic and triphasic OC also reportedly varies between brands [108]. The alternate types of OC may evoke different effects on exercise performance, particularly cardiovascular ability when exogenous estrogen is secreted in varying concentrations. Women taking monophasic OC are likely to exhibit consistent physiological adaptations due to a constant secretion of exogenous estrogen, compared with women taking biphasic or triphasic OC with progressive increases in exogenous estrogen secretion potentially affecting cardiovascular ability, and ultimately exercise performance.

Modern OC have considerably reduced the amount of synthetic hormones present compared with earlier generations of OC [115], with four- and ten-fold decreases in estrogen and progesterone respectively [37]. Despite the lower concentrations of estrogen and progesterone secretion in current-day (third generation) COC [37] compared with first-and second-generation OC, the relative secretions of both exogenous estrogen and progesterone differ from endogenous secretions throughout the normal menstrual cycle, with more progesterone and less estrogen secreted in COC compared with regular endogenous secretions [108]. Considering the concentration of estrogen and progesterone differ in OC users, it is probable that the effects of these hormones on the physiology and health of the woman would differ from normally menstruating women compared with women taking OC. Currently there is a lack of understanding with respect to the influence of exogenous estrogen and progesterone secretion on exercise capacity in OC users and this warrants investigation.

2. 2 .2 Combined Oral Contraceptives and Menstrual Disturbances

It is possible that highly active women who have been engaged in intense training regimes for substantial periods of time may use OC for cycle regulation [116], in the event that they are experiencing conditions such as secondary amenorrhea. In addition to regulating the menstrual

cycle, and despite the absence of conclusive evidence to substantiate the practice, COC are often used to treat dysmenorrhoea [117], with a noteworthy reduction in menstrual pain observed in COC users compared with placebo users [118] in women suffering from grade II or grade III dysmenorrhea as determined by the Andersch and Milsom dysmenorrhea index [119]. Further to dysmenorrhoea, COC may be useful in decreasing the symptoms associated with endometriosis, with reports of reduced pain in three out of four women taking OC [117, 120, 121]. A reduction in pain and symptoms associated with dysmenorrhoea and endometriosis in relation to OC use could be ideal for active women who may be experiencing these negative affects throughout exercise, and may contribute to the increasing rates of OC use in active women [122, 123]. Active women may also elect to take OC to avoid menstruation which can be done when women using COC continue to take active pills rather than only taking them for 21 days and then consuming the inactive pills which trigger menstruation [124-127], however taking OC to reduce pain and discomfort or to avoid menstruation may be unknowingly evoking adverse effects on exercise performance.

While the menstrual cycle does not appear to affect exercise performance [65-67] it is possible that the menstrual cycle could adversely affect perception of- and motivation towards exercise. Early studies [48, 115, 128, 129] investigating athlete attitudes towards competing at different stages of their menstrual cycles revealed a belief of performing 'best' during the inter-menstrual or postmenstrual phase and 'worst' in the premenstrual phase in Olympic athletes. Performance based studies in adolescent swimmers found [130, 131] the postmenstrual and menstrual phase to have the best performances, while the worst were observed in premenstrual phase or at the beginning of menses, which may also provide motivation for active woman to take OC as the timing of menstruation could be manipulated surrounding competition when the athlete believes she will perform optimally. Active women may also elect to use COC to reduce physical and psychological symptoms associated with PMS [132, 133]. Despite the reported benefit that OC may lessen the adverse symptoms associated with the menstrual cycle, women may dislike taking synthetic hormones particularly if they are not sexually active or if they are using other forms of contraception and deem the pill to be unnecessary. Conflicting findings regarding OC use and non-use in women who deem them unnecessary currently exist. In a survey of 461 female medical students 65.3% reported using OC regardless of their marital status, while 43.4% claimed they did not use OC because they were not necessary, suggesting the motives and deterrents for OC use are quite intricate and unique to the individual woman [134], and may therefore be influenced by other factors such as exercise level or knowledge of the effects of taking OC on exercise performance.

2. 3 PERCEIVED BENEFITS AND BARRIERS TOWARDS ORAL CONTRACEPTIVE USE IN WOMEN

2. 3. 1 Benefits of Combined Oral Contraception

Numerous non-contraceptive benefits of taking OC exist including decreasing uterine fibroids [135], relief of PMS [132], endometriosis [120, 121] and ovarian cysts [117] as well as decreasing the risk of some cancers including endometrial, colorectal and ovarian[117] . Oral contraceptive use may also be helpful in lessening the symptoms of dysmenorrhea [118], acne vulgaris [136], iron-deficiency anaemia [137] and may lead to increases in bone mineral density [111, 138]. Influencing the aforementioned menstrual symptoms may be of importance to highly active women to minimise the risk of fractures, particularly if they have been affected by menstrual disturbances causing changes in endogenous estrogen secretion throughout crucial periods of bone development. It not uncommon for females, particularly adolescent girls to be prescribed OC to help attenuate the symptoms associated with acne vulgaris [139]. Sex hormone binding globulin levels are elevated when using COC, subsequently decreasing ovarian and androgen hormonal secretion [117], which can help lessen hirsutism, acne lesion grade and count and seborrhoea [111, 136], which may be of psychological importance to female athletes, particularly if competition is televised and is highly publicised. In addition, COC use may help with menstrual disturbances as non-OC users were reportedly referred to hospitals presenting with excessive periods, painful periods, irregular periods or other menstrual disturbances more often than women taking COC [140].

The influence of OC was examined by a Cochrane review that was unable to demonstrate a reduction on menstrual blood loss in response to OC administration [141]. However an earlier study of 45 women concluded menstrual blood loss was lessened by 43% over two cycles in COC users when compared with non-users and progesterone-only users [137]. Estrogen may be the potential mechanism for reduced blood loss, because in women with high levels it has been observed to cause the inner lining of the uterus to thicken [43]. If estrogen is the major factor contributing to menstrual blood loss, this would explain why the recent Cochrane review [141] was unable to substantiate the earlier findings of Fraser and McCarron [137] as the relative concentrations in the OC administered in the Cochrane review [141], would have contained substantially less exogenous estrogen than the second generation OC likely investigated previously. The effect of OC on menstrual blood loss may be of particular importance to women suffering from menorrhagia (or menstrual blood loss of >80ml $cyle^{-1}$) which can pose a threat to

women as two-thirds of women diagnosed with the condition are also considered to have iron-deficiency anaemia [142]. Iron deficiency anaemia can place a serious health threat on women, which is why OC have often been prescribed to women to attenuate substantial menstrual blood loss [143]. Additionally, OC users have also been shown to exhibit elevated ferritin levels [144], and may therefore be able to store iron levels more effectively than non-users, which may have positive effects on bone development and health. It is therefore possible that OC secreting high dosages of estrogen may play a role in decreasing menstrual blood loss, which could be of importance to highly active women. Particularly women already at risk of iron-deficiency anaemia due to iron depletion from foot strike haemolysis and iron loss via sweating throughout exercise [145-147] or in women experiencing menorrhagia [148, 149] and could contribute to the increasing prevalence of OC use in modern-day, active women.

2. 3. 2 The Risks of Oral Contraceptive Use

In contrast to the reported health and wellbeing benefits of OC use in active women [117], [132] there are several risks associated with OC use, namely fluctuations in body weight [150, 151], breast tenderness [151], nausea [150], headaches [150] and interference with a woman's bleeding pattern, which is particularly prevalent in women using progesterone-only (mini) pills [43]. More serious health risks associated with OC use include increased risk of , ischaemic stroke [111, 152], breast [117, 138, 153] and cervical cancer [117, 138, 154], cardiac problems in smokers, myocardial infarction as well as a susceptibility to venous thromboembolism [111, 117, 155]. The risk of venous thromboembolism increases in women using OC however this increase is still considered minor [111], with reports of 5, 15 and 25 cases per 100 000 in non-OC users, second generation OC users, third generation OC users respectively [156]. Similarly, the risk of ischaemic stroke is slightly increased in COC users and this risk may be exacerbated in smokers or in women with hypertension [117]. Furthermore, the risk of cervical cancer is slightly elevated in COC users, with this risk rising with increased duration of use [111, 117]. However this time-dependent increased risk may not become pronounced until several years of OC use, with reports of users of five or less years not exhibiting any elevated risk compared with never-users, although COC users of eight or more years showed a very small increase in cervical cancer risk [157]. The increased risk of developing some cancers, venous thromboembolism, stroke or myocardial infarction, combined with non-life threatening factors such as nausea, headaches, breast tenderness and fluid retention may be considered barriers for OC use by some women, ultimately preventing them from taking OC.

The benefits associated with OC use appear to out-weigh the risks, with OC usage increasing in the Australian general population from 44.1%, 45% to 47.8% between 2005, 2008 and 2011 respectively [158], with similar usage rates reported in the Australian athletic population [159, 160]. It is possible, that women are not disregarding the health risks of OC use, rather this trend of increasing OC prevalence may reflect a lack of understanding of the way OC use may affect health and physiology, with women reportedly poorly informed about the effects of OC on health and performance [161-163]. Several survey-based studies have concluded [163-165] up to 75% of women are unaware of the health benefits of OC use not associated with preventing unwanted pregnancies. One study [166] found 54% of women believed there were substantial health risks associated with using OC, including cancer, heart attacks, blood clots and hypertension, with 40% of women unaware of any health benefits associate with OC use. However the respondents who were aware of the benefits of taking OC, reported cycle regulation to be the most important non-contraceptive benefit of the pill, followed by balancing hormone levels and a reduction in dysmenorrhoea. An earlier study [162] found only 42% of women did not believe there were non-contraceptive-related benefits to using the pill, while 22% stated cycle regulation as a supplementary benefit. A similar study to that of Bryden and Fletcher [162] of 1201 women from the UK, German and France aged between 16-45 years found cycle regularity, a reduction in dysmenorrhea and acne relief to be the most known, non-contraceptive benefits of the pill in 60%, 42%, and 37% of women respectively [163]. Correspondingly, in a study of 2248 ninth grade secondary school girls, menstrual cycle irregularity and treating dysmenorrhea were reported as the reasons for OC use, more often than preventing unwanted pregnancies, however the age of participants in this study should be considered as secondary school girls may not be sexually active and subsequently not using OC to prevent pregnancy [139]. It is possible a greater awareness of the relationship between OC use, health and exercise performance could affect OC usages rates in women and should be considered.

2. 3. 3 Oral Contraceptives and Athletic Performance

Oral contraceptives have been shown [7, 8, 167] to lead to a reduction of 5-15% in peak oxygen uptake ($\dot{V}O_2$ peak) and $\dot{V}O_2$ max, which could have significant implications for exercise performance. Females exhibit a smaller heart volume than males and consequently display reduced cardiac output ($\dot{Q}$) and stroke volumes [3], with $\dot{Q}$ considered a major contributor to $\dot{V}O_2$ max [168, 169]. Another important factor contributing to $\dot{V}O_2$ max is haemoglobin, specifically the concentration and number of red blood cells [5]. Generally women will exhibit a lower

haemoglobin level and total red blood cell count as a result of blood loss from the menstrual cycle [5]. It is therefore possible that this could be different between normally menstruating women and women taking OC, with OC shown to reduce blood loss associated with menstruation [137, 143]. Additionally, muscle repair and regeneration mechanisms were reportedly [170] less effective in COC users compared with normally menstruating women in response to controlled eccentric exercise-induced muscle damage. Together, these findings [7, 8, 170]·[5] suggest exogenous ovarian hormone secretion has a different effect on exercise performance than endogenous secretions.

Oral contraceptives are reportedly [35] used by more than 100 million women worldwide, with up to 76% of Australian women in 2001 reported to have used 'the pill' in the past [171]. Considering this high prevalence rate of COC use, it is not surprising that they are often used by active women. In 1997, 716 basketball, volleyball and soccer athletes were surveyed on women's health, finding 46% of athletes were taking some form of OC [122]. In a subsequent study of 112 American collegiate athletes, 44% reported taking some form of OC in 2006 [123]. Taken together these studies [123] [122] [35] [171] suggest OC use is increasing and depending on the age, demographics and socioeconomic status of women, OC use can be quite prevalent. Considering the growing number of women taking OC it is important to explore the way exogenous estrogen and progesterone secretion affects exercise tolerance and the health of the woman.

2. 4 OVARIAN HORMONES AND THE STRESS RESPONSE TO EXERCISE

2. 4. 1 Nervous System Activation and Cortisol Secretion

The HPA axis is crucial in the support of regular physiological functioning, in particular adapting to increased stress demands [172]. The hypothalamus receives and monitors information about the environment through various sensory means namely, visual, olfactory and hearing sensing as well as information regarding temperature, pain and emotion [45]. Upon receiving this information, appropriate responses will be coordinated by the hypothalamus through neural stimulation and hormone release, in an attempt to maintain homeostasis [45]. Specifically, upon activation of the HPA axis, CRH neurons are sent regulatory impulses from several neurotransmitter systems including β-endorphin-producing neurons [173-175] and pro-inflammatory cytokines to initiate CRH secretion, stimulating the release of ACTH from the pituitary gland [172, 176, 177]. At this point the adrenal cortex secretes glucocorticoids [178],

namely adrenalin and cortisol [177, 179, 180]. Cortisol is a very potent hormone in human physiology and can easily target most of the body's cells [181]. Cortisol is responsible for several catabolic processes including reduced protein synthesis, increased protein degradation and inhibition of the inflammatory process [182, 183]. Additionally cortisol plays a role in stabilising metabolism and energy production [172, 184] by maintaining glucose production from protein, facilitating adipose metabolism and supporting vascular responsiveness, as well as regulating several important physiological systems, including the central nervous system (CNS), the cardiovascular system and the immune system [172, 185, 186]. Although the menstrual cycle does not appear to influence the HPA axis [139, 302, 318], the variation in the concentration of exogenous estrogen and progesterone secreted while taking OC may evoke a different HPA axis response and should be investigated as it remains largely unexplored.

2. 4. 2 Cortisol and the Hypothalamic-Pituitary-Adrenal Axis

An increase in the amount of circulating cortisol which is commonly associated with HPA axis activation and subsequent secretion of adrenal glucocorticoids, often results in a negative feedback loop which leads to HPA axis suppression [187] through the increased release of anti-inflammatory cytokines [188]. Chronic stress has been shown to cause changes in the HPA axis, ultimately leading to circadian changes in cortisol secretion [189, 190] and a decreased sensitivity of cortisol [191] which may lead to higher circulating concentrations of cortisol. Prolonged increases in the levels of circulating cortisol due to chronic activation of the HPA axis could lead to reduced sensitivity of the hypothalamus and the pituitary to glucocorticoid signalling, which inhibits the negative feedback loop designed to reduce HPA axis stimulation [187]. The prolonged release of glucocorticoids from chronic HPA axis stimulation may adversely affect the immune system by decreasing the number and function of several circulating immune cells including lymphocytes, reducing antigen presentation [177] and can lead to degenerative health potentially exacerbating existing health problems [45]. Intense or prolonged exercise which elicits a greater stress response and subsequent release of cortisol, could ultimately contribute to the suppression of the immune system, which may be amplified in women taking OC due to the association between exogenous estrogen and increases in cortisol concentrations through amplified HPA axis activity [192]. If women taking OC are experiencing greater cortisol concentrations as a result of an estrogen-induced amplification of HPA axis activity, they may experience further reductions in immune functioning, ultimately predisposing them to illness.

2. 4. 3 The Effect of Estrogen on the Hypothalamic-Pituitary-Adrenal Axis

Proposed changes in mineralocorticoid and glucocorticoid receptor gene expression and function have been suggested to be responsible for HPA axis stimulation in humans and animals [193-196]. Additionally, estradiol-induced impairment of negative feedback regulation [194], changes in the electrical and chemical characteristics of hypothalamic neurons [197] and improved CRH gene transcription in the hypothalamus as a result of estrogen [192] have also been suggested as possible mechanisms of increased HPA axis activity. Changes in the suprachiasmatic nucleus (SCN) which is involved in the daily regulation of cortisol secretion, in response to estrogen levels during the menstrual cycle may also contribute to variations in HPA axis activity [198, 199]. However, as the HPA axis was not shown to be affected under control or challenge conditions throughout the menstrual cycle [19, 200, 201] and no evidence of daily fluctuations in HPA axis activity were found as a result of the menstrual cycle in healthy women [202, 203], it is possible that only exogenous estrogen in OC may have a pronounced effect on the SCN.

2. 4. 4 Exercise and Stress

Stress is the state in which under the influence of internal or external stimuli (stressors), homeostasis is threatened [204]. The core stress response, which was originally described by Selyne in 1936 [205, 206] indicates the acute production of glucocorticoid hormones from the adrenal cortex stimulates the metabolism of glucose to produce energy, which is used to evade an immediate threat, through the fight or flight response [207, 208]. Any disturbance of the stress response can result in an imbalanced physiology of the body, thus leading to enhanced susceptibility to infection or inflammatory disease [45]. There are several stressors which can elicit a cortisol response from the HPA axis. Cognitive or psychological stress is reportedly sufficient to lead to increases in cortisol levels [209], as well as the threat of physical danger [210] and ambient temperature [211]. A potent stressor is exercise, such as that undertaken by participants in the present thesis, as it causes substantial changes to the physiology of the organism and threatens homeostasis [212]. Increases in the concentration of stress hormones such as cortisol [213-215] have been reported to occur both during [216] and prior [217] to exercise as a result of physical exertion and anticipation of exercise.

2. 4. 5 The Effect of Estrogen and Progesterone on Cortisol

The effect of endogenous ovarian hormonal fluctuations on circulating cortisol levels is equivocal [19, 200-202, 218, 219]. With no differences observed between the plasma adrenocorticotropic

hormone (ACTH) and cortisol concentrations in the luteal and follicular phases of the menstrual cycle [21, 201, 219, 220] when challenged under high intensity (90% $\dot{V}O_2$ max) and endurance conditioning. While others have shown [19, 20] cortisol and ACTH levels to be decreased throughout the follicular phase of the menstrual cycle and increased during ovulation, at rest. Exogenous ovarian hormones in the form of OC appear to have clearer effects on circulating cortisol when compared to endogenous secretions. Women using COC reportedly exhibit increased resting levels of serum cortisol compared with non-contraceptive users and males [21, 221, 222]. The elevated serum levels of cortisol which were observed [221, 222] in women using COC, may be a result of ethinyl estradiol which is a common estrogen found in monophasic OC. Ethinyl estradiol reportedly increases the production of hepatic corticosteroid-binding globulin (CBG) [19, 21, 223], leading to increases in the binding capacity of serum cortisol as well as the total concentration of cortisol and can potentially prevent connection of cells to the endothelium by influencing neutrophil trafficking [224]. In addition to increasing the binding capacity and total concentration of cortisol, an increased synthesis of CBG observed in OC users [19, 21, 222, 223], results in lower metabolic clearance rates and larger half-life times for cortisol metabolism. Importantly, this increase in CBG-bound cortisol levels, appears to act as a buffer within the plasma, ultimately delaying HPA axis stimulation [222], consequently affecting normal circadian fluctuations in cortisol and potentially dampening the free cortisol response [19]. Long-term exposure to elevated cortisol levels can have adverse effects on the body including muscle atrophy, impaired growth and tissue repair and immunosuppression as well as causing changes to normal circadian rhythms [225-227]. Furthermore, when cortisol levels are extremely high cognitive processing can be adversely affected [228], which could pose complications for not only competitive exercise, but activities of daily living. On the basis of our current understanding of the influence of long term elevated cortisol levels associated with OC use it is possible that exogenous estrogen and progesterone administration may have adverse consequences for the health and performance of active women and warrants further investigation.

2. 4. 6 Circadian Variations and Cortisol Secretion

Cortisol has been shown to experience a circadian rhythm throughout the day [176] which may affect the secretion of cortisol in response to exercise throughout a 24 hour period [229]. Cortisol secretion rates are reportedly elevated after morning awakening, generally peaking within 30 - 45 min post-awakening and progressively declining throughout the day [22]. It is this response to consciousness which is referred to as the cortisol awakening response (CAR) [230, 231]. The

CAR is a component of physiology closely regulated by the circadian cycles which are necessary for homeostasis and healthy living; it is not an isolated response to morning awakening [181]. Pruessner et al (1995) found a 50-100% increase in salivary free cortisol within 30 min of awakening in healthy participants [232], highlighting the demand for standardisation of testing protocols and caution when interpreting early morning samples [233]. Circadian variations in salivary cortisol appeared to be affected throughout the various stages of the normal menstrual cycle, with minor variations in salivary cortisol occurring in response to the various phases of the cycle [234, 235]. However, the CAR was neglected in both of these studies [234, 235] and the ovulatory phase was excluded from Odber et al (1998) [234]. It has been reported [46] that the CAR may be affected during ovulation due to gonadal steroids stimulating HPA axis regulation, and any variation in results during this period (cycle days 12-16) may reflect changes in hormonal levels. This variation in hormone levels which may be stimulating the HPA axis may be different between OC users and normally menstruating women due to the variation in concentrations of estrogen and progesterone secreted throughout either cycle. Suggesting differences in cortisol section may occur between women taking OC and women who are normally menstruating.

2. 4. 7 The Effect of Exercise on Cortisol Secretion

The intensity and duration of exercise may determine the magnitude of the stress response to exercise, with cortisol expected to rise sequentially with the duration of physical activity [236]. High intensity, acute resistance exercise has been shown to result in a 97% increase in salivary cortisol from baseline values [237], with increases in cortisol concentrations also observed following wingate sprint interval training in recreationally healthy males [238], a 6 hr relay race in healthy males [239] and high intensity cycling lasting 1hr in duration [240]. Once exercise reaches 60-65% $\dot{V}O_2$ max the HPA axis becomes stimulated and increases in T_C and the concentration of several stress hormones including cortisol, adrenalin, growth hormone and β-endorphin increase [241, 242], with the release of several of these stress hormones including adrenalin leading to increases in HR and blood flow to lymphoid tissues [242]. The intensity and duration of exercise may influence the extent of the cortisol response with serum levels of cortisol reported to remain elevated for several hours following the cessation of endurance exercise [243-245]; indicating prolonged exercise has a more pronounced effect on serum cortisol compared to acute exercise. Additionally, the type of exercise undertaken may contribute to the cortisol stress response to exercise with cortisol observed to reach extreme values in elite rowers, which may be due to the large muscle mass utilised throughout rowing [246, 247], suggesting the volume of

muscle mass used throughout exercise may influence the subsequent stress response. Cardiovascular capacity may also contribute to the subsequent cortisol response to exercise, with highly trained individuals often evoking an increased cortisol response to exercise, compared with less trained individuals [248, 249]. It is possible that individuals with greater cardiovascular capacities may be able to sustain a greater metabolic demand for a longer period of time compared with less trained individuals, which may ultimately lead to a greater secretion of cortisol and adrenalin following exercise. The increased metabolic and thermoregulatory demands placed on the individual when exercise intensity increases and/or a greater muscle mass is recruited, may contribute to the exercise-induced increase in cortisol secretion often observed following exercise cessation. This increase in metabolic and thermoregulatory demand could be exacerbated in women taking OC if they are experiencing chronically elevated T_C and cortisol secretions due to exogenous secretions of estrogen and progesterone. If exogenous estrogen and progesterone are contributing to an increase in exercise-induced cortisol levels it is important to explore if exercise performance is negatively affected, particularly in the context of female athletes, as this may ultimately affect their decision to continue or discontinue OC use.

2. 4. 8 Physiological Role of Cortisol

Cortisol can have both anabolic and catabolic effects on various parameters of human physiology [250]. In an anabolic manner, cortisol increases hepatic gluconeogenesis [250], a metabolic pathway which produces energy from non-carbohydrate substances. The catabolic actions of cortisol include a breakdown of tissue (adipose and lean) for energy production, and can inhibit the growth of connective tissue [250]. Several immune cells such as lymphocytes, granulocytes and monocytes and macrophages exhibit glucocorticoid receptors which bind cortisol [45, 251] and subsequently reduce the regulation of cytokine-producing cells, through the inhibition of NF-$_K$B [45]. The suppression of NF-$_K$B causes a change in gene expression and deregulates normal immune functioning [45], causing changes in cellular trafficking and proliferation, antibody production, as well as impaired effector functions of lymphocytes [252, 253]. In particular, a major inhibitory effect of elevated cortisol is decreasing transepithelial transport of s-IgA and in vivo B-lymphocyte antibody synthesis [24, 254-256], as well as being linked to decreases in B-lymphocyte mobilisation [24] following exercise. If women taking OC are exposed to greater concentrations of cortisol post-exercise, they may be at risk of compromising immunity. Subsequently at risk of illness and infection, which may adversely affect exercise performance in active women and the ability to compete in female athletes.

2. 5 THE IMMUNE SYSTEM AND EXERCISE

2. 5. 1 The Structure of the Immune System

The immune system is designed to protect the organism from pathogens including viral and bacterial infections which may compromise homeostasis and subsequently health. The common mucosal immune system is a key self-defence mechanism and it exists to prevent harmful pathogens and antigens from entering the organism. The common mucosal immune system connects a number of immune structures throughout the body to protect the host from antigen presentation [257], namely the gut, urogenital tract, oral cavity and respiratory system [258]. The mucosal immune system is the first barrier to infection by microorganisms and protects the surfaces of both the respiratory and gastrointestinal tracts [259].

2. 5. 2 The Structure of the Common Mucosal Immune System

Two components of the immune system unite to form the structure of the common mucosal immune system, namely the innate immune system and the adaptive immune system, each of which play a crucial role in immune system processing. The innate immune system is comprised of several anatomic and physiological components including mucous membranes and is considered the first aspect of the immune system to fight infection [260, 261]. The adaptive immune system is responsible for the proliferation of T- and B-cells [262], which produce plasma cells and eventually antibodies, such as s-IgA [263]. Once a B-cell receptor comes into contact with an antigen, daughter B- and T-cells are created which bind specifically to the antigen [263]. The daughter B- and T-cells can multiply into plasma cells and produce Immunoglobulins such as antibodies or form memory cells which will identify the antigen more efficiently upon secondary contact [263]. Common actions of immunoglobulins include binding to antigens on microorganisms, neutralizing bacterial toxins, inhibiting bacterial and viral movement and entry into host cells, as well as facilitating cytotoxicity by T_C and NK cells and phagocytosis against toxins [264].

2. 5. 3 Salivary Immunoglobulin A

Salivary immunoglobulin A is considered one of the most abundant proteins in the saliva [265] and is often monitored in applied sport science research as athletes experiencing a reduction in [s-IgA] below 40% of their mean values were reported to have a 50% chance of developing an URTI within three weeks [31]. There are several protective functions of s-IgA including preventing antigens and microbes including influenza from adhering to and penetrating the

epithelium (immune exclusion), preventing replication of intracellular pathogens during transcytosis through epithelial cells (intracellular neutralization), and binding to antigens in the sub-mucosa, facilitating their evacuation from the lamina propria via a secretory component (SC) through the epithelium and back to the lumen (immune excretion) [25, 258, 266-268]. Even though the initiation of up to 95% of all infections occurs at the mucosal surfaces in which s-IgA is the most abundant immunoglobulin [269, 270], decreases in [s-IgA] do not solely equate to infection , rather the [s-IgA] on mucosal surfaces is an indicator of the potential risk of infection [271]. However it does provide a convenient method of monitoring the health of an individual, particularly in response to exercise when venepuncture is not always accessible or sanitary.

2. 5. 4 The Structure of Salivary Immunoglobulin A

The base structure of immunoglobulins consists of four polypeptide chains comprised of two identical light chains (MW 25 kDa) and two identical heavy chains (MW 50-77 kDa) [177, 264, 272, 273]. The light and heavy chains are bound together by both covalent disulphide bridges and non-covalent interactions [177]. The light chains are generic among immunoglobulins; however variations in the heavy chains occur depending on their specific immunoglobulin class [264, 273, 274]. Heavy and light chains exhibit both variable and constant regions, which consist of the region containing the antigen-binding site and the region determining the fate of the antigen, respectively [177, 273-275]. Immunoglobulins are bi-functional in nature, with different functions at opposing ends of the molecule [264]. The fragment crystallized (Fc) (55 kDa) [275] portion stimulates several aspects of the immune response by binding to complement and Fc receptors on the antigen-antibody complex [177, 264]. The opposing end of the immunoglobulin is responsible for antibody binding and is made up of two fragment antibody binding (Fab) segments (MW 45 kDa) [177, 264], [275]. The molecular mass of the two IgA monomers, the joining (J)-chain and the SC which join to form a single polymeric molecule [177, 272, 276, 277], is 300 000, 15 600 and 70 000 Da respectively.

The assembly of an s-IgA molecule includes the fundamental immunoglobulin structure [278] as well as a J-chain and a SC which acts to construct a polymer from multiple monomers [279], as well as stabilising and protecting the IgA polymer from breakdown. The J-chain is synthesised in plasma cells, while the SC is produced by mucosal epithelial cells and both are disulphide-linked to the Fc region of the IgA molecule [276, 279]. The SC acts as a s-IgA receptor, while stabilising the structure of polymeric IgA protecting IgA from protein breakdown when secreted into saliva

[177, 279, 280]. When the SC is membrane-bound it is referred to as a poly-immunoglobulin receptor (pIgR) and acts as receptor for transepithelial transport of polymeric IgA (pIgA) [278, 281]. The expression of the pIgR has been reportedly altered in response to intense exercise [282] and the secretion of estrogen [283], which could contribute to exercise-induced suppression of [s-IgA] in active women and may explain why female athletes exhibit lower concentrations of s-IgA [284-286].

2. 5. 5 The Production of Salivary Immunoglobulin A

Salivary immunoglobulin A is produced by plasma cells situated nearby the ducts and acini of the salivary glands, with each salivary gland secreting a unique concentration of cells depending on its nature [287]. Acinar cells can be divided into two forms, namely serous and mucous cells [288]. Serous acinar cells are located in the parotid gland and secrete a thin, watery and amylase-rich form of saliva through a excretory duct into the oral mucosa [288], whereas both serous and mucous acinar cells are present in the submandibular gland, producing a viscous and mucin-rich saliva, which is secreted into the sublingual mucosa [288]. Each salivary gland contributes varying amounts of saliva into the oral cavity which highlights the demand for standardisation when collecting saliva; if samples are collected from different parts of the oral cavity the respective concentrations of proteins as well as secretion rates can vary and lead to a misinterpretation of mucosal immune status.

The process of s-IgA production involves the contribution of the pIgR, the J-Chain, specific endosomes and the SC, to transport pIgA to mucosal surfaces, including the upper respiratory tract [259, 289, 290]. Polymeric IgA is transported to mucosal epithelial cells through the transmembrane glycoprotein pIgR [279, 291]. The pIgR is synthesised in the rough endoplasmic reticulum and then transported to the Golgi apparatus [291] where it is divided into vesicles before travelling to the basolateral surface of epithelial cells [291]. At the basolateral surface of epithelial cells [275], the pIgR recognises and binds to J-containing pIgA [276] to form the pIgR-pIgA complex [259, 292]. The pIgR-pIgA complex undergoes endocytosis and is transported to basolateral early endosomes [272, 279, 291], where it is secreted through a common endosomal compartment to reach the apical membrane [275]. At the apical membrane the pIgR-pIgA complex is broken down through proteolysis into apical recycling endosomes (ARE) [291, 293]. Once broken down, pIgR binds extracellular, leading to the secretion of s-IgA [294] and the SC as either part of the s-IgA complex or in free form [291]. Once the SC has been released, portion

of the pIgR can be re-utilised as an apical early endosome (AEE), and recycled back to the apical surface by ARE [291]. Polymeric-immunoglobulin receptors are continuously transported independent of pIgA binding, when this occurs, any unoccupied receptors undergo proteolysis, and the SC is released [266]. The SC is synthesised in epithelial cells, which line the mucous membranes and exocrine glands [276, 290], not plasma cells. The SC was observed to be a proteolytic fragment of the pIgR [295] and only pIgA which exhibits a J chain can bind to the surface of pIgR containing epithelial cells [296]. In s-IgA, there is a 1:1 stoichiometry between the SC and dimeric IgA, with epithelial cells synthesising one pIgR particle for every one transported.

2. 5. 6 Initiating the Production of Salivary Immunoglobulin A

Two possible mechanisms have been suggested to initiate the production of s-IgA; the local response theory and the common mucosal immune system theory [292]. The local response theory suggests that the proliferation and differentiation of the lymphoid cells in the duct-associated lymphoid tissue (DALT) near the salivary glands are stimulated by the presence of oral antigens [292]. The common mucosal immune system theory is based on the influx of antigen-susceptible IgA precursor B-cells from mucosa-associated lymphoid tissue (MALT) to the salivary glands [292]. When the MALT comes into contact with an antigen, IgA B-and T-cells exit into the circulation, where they target the lamina propria of the intestine, lungs and exocrine glands i.e. salivary glands [277, 292]. Once the IgA B-and T-cells reach the mucosal and glandular tissues, they grow and mature into IgA plasma cells [278] and are secreted. A number of other factors have been shown to initiate the production and release of s-IgA. Importantly, the nervous system has also been shown to increase the secretion of s-IgA into saliva through the stimulation of the sympathetic and parasympathetic nervous systems [297, 298]. The autonomic nervous system supplies nerves to the immune cells in lymphoid organs and has been shown to induce changes in local immune processing [299]. In particular, the autonomic nervous system has been shown to enhance the transfer of s-IgA [300], however in vivo studies have not conclusively proven it to enhance the release of IgA [300]. It is possible that there are several mechanism for which s-IgA is produced, each of which could be affected by external factors such as circulating hormone levels as well as exercise-induced stress.

2. 5. 7 Exercise and the Immune System

The number and function of several cells of innate and adaptive immunity are affected in response to exercise [301]. Several changes in the functioning of the adaptive immune system have been observed in response to exercise lasting 90 min or greater including decreases in [s-IgA] as well as decreases in B- and T-cell numbers, proliferation and function [30, 302-305]; these changes lead to impaired cell-mediated immunity [30]. The extent of which the mucosal immune system is affected by exercise appears to be directly related to the volume and intensity of exercise [301, 306-308]. Moderate intensity exercise of between 40-60% $\dot{V}O_2$ max appears to stimulate immune functioning, through increases in [s-IgA] and secretion rates [264, 309-311], whereas decreases in nasal and salivary IgA concentrations [312] and the proliferative responses of lymphocytes to mitogens [261] have been observed following intense exercise between 55-80% $\dot{V}O_2$ max, further underlining the relationship between exercise intensity and immune system function.

Several studies have investigated the s-IgA response to exercise over various training intensities, concluding the greatest immune response was observed throughout endurance training in moderately active runners and elite swimmers [313, 314], with shorter, intense periods of training showing less substantial changes in mucosal immunity [315, 316]. Prolonged, heavy exertion has been shown to decrease [s-IgA] by 9.3 and 5.5% in runners [317] and swimmers [310] respectively. To further demonstrate the association between exercise intensity and the s-IgA response [318, 319], up to a 65% decrease in [s-IgA] was observed following intense cycling [320], while little or no change in [s-IgA] or output was observed in response to low or moderate intensity swim training [321], tennis drills [322], acute and prolonged weight training [323]. Further decreases in [s-IgA] have also been reported following kayaking [306], rowing [307, 324], hockey [325], interval exercise on a cycle ergometer [326], repeat sprint cycling (Wingate tests) [238, 327], basketball [328] and running [302, 316, 321, 329]. Similarly, moderate exercise (greater or equal to 3.0 metabolic equivalent (METS)) in non-athletic, healthy adults was reported to decrease the annual risk of URTI by 20-30% [330], which is indicative of the 'J-curve' theory suggested by Nieman [331]. The J-curve theory suggests that infection risk is directly proportionate to the amount of exercise undertaken as well as the intensity and duration of the exercise, regardless of the population. The J-curve theory suggests that both low and high levels of physical activity are associated with an increased susceptibility to illness, while a moderate level of physical activity is associated with a reduced risk of URTI [331-333]. The J-curve theory

is of particular importance when considering recreationally active women rather than sedentary women or female athletes, as the exercise-induced stress and mucosal immune response should be relatively unaffected and up-regulated respectively. However if the recreationally active woman is taking OC, it is possible that a greater exercise-induced stress response may occur, even following moderate intensity activity, which may have consequences for the mucosal immune system, including decreases in [s-IgA], which may have otherwise led to a surge following the same exercise session.

2. 5. 8 Exercise and the Production of Salivary Immunoglobulin A

Intense exercise has been reported to adversely affect the pIgR by decreasing messenger ribonucleic acid (mRNA) expression in the submandibular gland [282]. Interestingly, moderate exercise has been suggested as an up-regulator of pIgR production [334], as a result of nervous system stimulation and subsequent changes in hormone secretion [256]. If pIgR expression is increased, the ability of mucosal epithelial cells to transport dimeric IgA will be enhanced [291]. Additionally, several pro-inflammatory cytokines which are synthesised upon viral and bacterial invasion, contribute to the up-regulation of pIgR expression [291]. If the transcytosis of pIgA to pIgR is enhanced as a result of pIgR up-regulation throughout moderate exercise, pathogen and antigen neutralization may occur more readily [291].

2. 5. 9 The Effect of Cortisol on Salivary Immunoglobulin A

Cortisol has been reported to adversely affect the immune system by impeding the synthesis of B-lymphocytes [254-256, 335], and in particular by down-regulating the production and transport of s-IgA [24, 336]. Several authors [310, 337] have reported increases in cortisol as a result of acute, prolonged or intensive training did not cause changes in post-exercise [s-IgA] in swimmers or runners. The effect of the physical stress associated with exercise on s-IgA remains equivocal, despite a known association between psychological stress and the suppression of s-IgA [338, 339]. Given the established influence of psychological stress on sCort levels, it is possible that the type of stress leading to the secretion of cortisol, may contribute to the subsequent effect on mucosal immunity.

2. 5. 10 The Effect of Carbohydrate intake of Salivary Immunoglobulin and Cortisol

Dietary patterns may influence the immune response to exercise as several components of the immune system require nutrients and vitamins for normal functioning [340]. Glutamine is well

established as an energy source for the cells of the immune system, however it has now been identified as an important fuel for lymphocytes [340]. During physical exercise, muscles and organs require increasing levels of glutamine to function, which may lead to glutamine debt, ultimately compromising the availability of glutamine for energy demands [340]. Even though a recent study was unable to conclude an association between post-exercise decline in immune function and decreased plasma glutamine concentrations [29], a reduced incidence of URTI following marathons in trained runners occurred following the ingestion of beverages containing glutamine [341]. In addition to reducing the incidence of URTI, carbohydrate (CHO) ingestion has been suggested to assist in maintaining plasma glucose concentrations, consequently reducing the stress hormone response to exercise [29]. Several studies have investigated the use of CHO beverage consumption before, during and after 2.5 hr of exercise concluding higher plasma glucose levels resulted in a reduced cortisol response, lower granulocyte and monocyte phagocytosis and oxidative burst activity, and a diminished pro-and-anti-inflammatory cytokine response [342-344]. It is therefore possible that fuel availability may also contribute to the stress and mucosal immune response to exercise.

2. 5. 11 Circadian Variations in Salivary Immunoglobulin A

Circadian rhythms have been reported to affect saliva flow rate, with the highest flow being reported at 0400 hours and the lowest observed at 2000 hours. It is vital that sample collection procedures are standardised to account for circadian rhythms and do not induce subsequent stress on the participant, as this can lead to distorted results [233]. The effects of circadian variations on [s-IgA] have been investigated [24, 345], with varying results. Dwyer, et al (2010) reported no evidence that [s-IgA] was higher in the morning or evening [345], whereas Hucklebridge, et al (1998) and Gleeson et al (2001) reported [24, 346] [s-IgA] was higher in the morning but slowly decreased until a plateau was achieved approximately six hours after awakening. It is possible that saliva flow rate may have contributed to the discrepancies between findings, with dehydration following overnight fasting reducing the flow rate of saliva into the oral cavity; subsequently [s-IgA] appears to be higher. In addition to dehydration from overnight fasting, oral drying as a result of high environmental temperatures may cause variations in saliva flow rate, and should be considered when interpreting results in hot and humid (HH) conditions.

2. 6 OVARIAN HORMONES AND THE IMMUNE RESPONSE TO EXERCISE

2. 6. 1 The Effect of Estrogen and Progesterone on the Immune System

Lymphocytes exhibit specific receptors to which estrogen and progesterone connect, namely the ERα and ERβ, as well as the progesterone receptor (PR) [32, 38, 347]. These ovarian hormones influence cell responses by connecting to nuclear and non-nuclear receptors [348], with estrogen largely shown to enhance immune response and progesterone reported to reduce immune function [57-60, 349]. Specifically estrogen has been shown to affect B-cell development leading to increased concentrations of immunoglobulins [9, 350] as well as regulating the T-helper 1 (TH1) and T-helper 2 (TH2) response, with low levels of estrogen invoking TH1-mediated immune response and high levels of estrogen leading to TH2-mediated immune response [351]. Estrogen is a key contributor to maintaining normal immune function in women [33, 107]. A significant positive linear correlation was observed between estrogen and [s-IgA] in healthy pre-menopausal women [352], with the pattern of [s-IgA] mimicking serum estradiol through the various phases of the menstrual cycle [34, 57, 58]. Additionally the decreasing [s-IgA] observed throughout the postovulatory phase coincided with decreases in estradiol in parotid saliva [34], reinforcing previous research concluding elevated estrogen levels were associated with enhanced immune function [353]. The phase of the menstrual cycle, combined with exercise-induced changes, may further augment the risk of URTI in women i.e. during the follicular phase of the menstrual cycle when estrogen secretion is lower, and consequently the production of s-IgA appears reduced. The effect of exercise on [s-IgA] throughout the follicular phase of the menstrual cycle should be investigated to establish if women experiencing low secretions of estrogen are amplifying exercise-induced decreases in mucosal immunity.

2. 6. 2 The Effect of Estrogen and Progesterone on Salivary Immunoglobulin A

The normal menstrual cycle has been observed to influence the release of the SC which is an imperative part of s-IgA production [283], when estradiol levels are elevated [34]. Specifically, estradiol is reportedly [34] the predominant sex-steroid hormone which controls the synthesis and release of the SC, which could explain its profound effect on [s-IgA]. Additionally, salivary estradiol, is reportedly [352] significantly correlated with salivary IgA in healthy women, and the pattern of serum estradiol mimics [s-IgA] through the various phases of the menstrual cycle [34], further demonstrating the relationship between estrogen and s-IgA. While the mechanism remains equivocal, estrogen and progesterone have been shown to affect the expression of the pIgR throughout the oestrous cycle in humans [283] and rats [354]. Saliva composition and secretion

into the oral cavity are also thought to be affected by estrogen receptors, which are located in the oral epithelium and salivary glands [355]. Estrogen may therefore play a role in s-IgA regulation, by modulating the expression of the SC and pIgR as well as saliva secretion. Despite the association between s-IgA and endogenous estrogen throughout the menstrual cycle [33, 107], less is known about the relationship between exogenous ovarian hormones and s-IgA production. It is however possible that women taking monophasic OC may experience increases in [s-IgA] more consistently than women who are normally menstruating due to the constant supply of exogenous estrogen throughout the active pill phase. Despite the constant secretion of estrogen in COC particularly monophasic preparations, the total amount of estrogen secreted remains lower then throughout the normal menstrual cycle, which may explain why women taking OC experienced an 82 and 38% decrease in lymphocytes following cycling for 90 min at 65% $\dot{V}O_2$ peak when compared to male controls [221, 356], whereas no significant changes in leukocytes, neutrophils, and monocytes were observed in normally menstruating women in response to running [357, 358] and cycling [359, 360] when compared to male controls. Together, these studies [352, 357, 358] suggest that endogenous and exogenous estrogen secretion may have different effects on [s-IgA] in healthy pre-menopausal women and investigation into the effect of OC on [s-IgA] in response to exercise should be conducted to identify if exogenous and endogenous secretions of estrogen evoke different responses from the mucosal immune system.

2. 7 OVARIAN HORMONES AND THE CORE BODY TEMPERATURE RESPONSE TO EXERCISE

2. 7. 1 Core Body Temperature in Humans

Core body temperature is regulated at a 'set-point' of approximately 37°C in humans [361] and maintains stability due to nerve cells located within the hypothalamus [361] while the body is at rest. Throughout exercise T_C will rise proportionately alongside exercise intensity, with this increase in T_C augmented in HH conditions [361, 362]. Environments with high humidity cause further issues specifically for thermoregulation, as the ability for sweat to evaporate is drastically reduced [362]. When heat dissipation is occurring at the same rate as heat generation a plateau in T_C occurs [361, 362], which enables the individual to sustain the metabolic and thermoregulatory demands of exercise without risking premature fatigue. Interestingly, throughout steady state exercise, the rise in T_C has been suggested to occur proportionally to metabolic rate, rather than as a result of ambient temperature [363]; consequently, if women taking OC are experiencing a

higher metabolic demand than normally menstruating women in response to exercise it is possible they may experience a greater increase in T_C.

2. 7. 2 The Effect of Exercise on Core Body Temperature

Increased muscular contractions which occur throughout exercise produce heat from excess energy production to meet the metabolic demands of exercise [362]. Throughout the initial stages of exercise heat generation often exceeds heat dissipation [361]. When this occurs, a small amount of the heat generated from exercise is lost through the skin [361], however the majority of the heat returns to the core of the body via a convective gradient of venous blood returning to the heart [362], ultimately causing an increase in T_C. As T_C continues to increase, central thermo-detectors located within the hypothalamus and spinal cord recognise the increasing heat needs to be reduced, and subsequently initiate thermoregulatory mechanisms, including increasing cutaneous vasodilation and sweating [361]. Exercise-induced rises in T_C may be influenced by environmental conditions, with higher environmental temperature likely causing more rapid increases in internal temperature [362]. Investigations into the T_C response to exercise must include both TN and thermally-stressful i.e. hot conditions to examine the role of the external environmental on internal temperature regulation.

2. 7. 3 Core Body Temperature and Exercise Performance

Heat stress has been shown to result in reduced stroke volume and increased HR in response to moderate-intensity exercise [364, 365], which compromises $\dot{Q}$, ultimately affecting exercise performance [365]. A compromised $\dot{Q}$ may explain why exercise time to exhaustion is reportedly influenced by the degree of change in T_C from pre-exercise levels [366-369]. Monitoring T_C throughout exercise, particularly in HH conditions is crucial as elevations in T_C above an optimal level can negatively affected exercise performance [361]. When T_C increases, blood flow distribution is placed under substantial demand to maintain the metabolic, cardiovascular and thermoregulatory requirements of exercise [361]. If any of the aforementioned parameters are compromised, it may substantially affect the ability to perform exercise, and may lead to premature fatigue [361]. Rising T_C has been reported to contribute to premature fatigue throughout exercise [361, 364], by increasing the ratio of α / β waves, which contribute to a reduced alertness and arousal throughout exercise [361]. This reduction in alertness and arousal negatively affects the motivation to continue exercising [361] and is considered a defence mechanism which is triggered when T_C exceeds an optimal and safe level [370]. In addition to

changes in the ratio of α / β waves, a reduction in cerebral blood flow may occur when T_C is high, also causing further changes to alertness and arousal and potentially contributing to fatigue[370]. Further to changes in the ratio of α / β waves, cardiovascular drift has been shown [371-373] to have a plausible causal link with a decreased $\dot{V}O_2$ max during heat stress. With dehydration considered another contributor to premature fatigue while exercising in hot conditions through reduced locomotor muscle blood flow, oxidative metabolism and $\dot{Q}$ [374]. Several authors [375, 376] have proposed the T_C in which exercise performance is affected and fatigue is experienced approaches 40°C, however in untrained individuals, a T_C approaching 38°C is sufficient to cause exhaustion [377-379]. Establishing at what point T_C initiates these anticipatory defence mechanisms, which may be contributing to premature fatigue throughout tolerance exercise, could prove beneficial for the management of active women and female athletes and reduce the risk of premature fatigue throughout exercise.

2. 7. 4 The Effect of Estrogen and Progesterone on Core Body Temperature

The role of endogenous estrogen and progesterone on resting T_C is well established [9, 10, 12-15], with progesterone shown to increase basal body temperature by 0.3°C - 0.5°C [380-382] during periods of high secretion, while estrogen reportedly attenuates this effect subsequently lowering T_C [14, 15, 383, 384]. Estrogen and progesterone interact in an antagonistic manner, and when both hormones are secreted simultaneously, the effect of progesterone appears to surpass estrogen [380, 385-387], thereby resulting in increases in T_C. Endogenous secretions of estrogen and progesterone are known to influence T_C and the $T_{C\ THLD}$ for cutaneous vasodilation [12-14] by modifying warm and cool sensitive neuron activity [14, 383, 386, 388]. It is therefore not surprising that fluctuations in T_C occur throughout the various phases of the menstrual cycle. Some forms of hormonal contraception, including the injectable progesterone method, have been shown to cause increases in T_C throughout the 24-36 hr period following administration [389]. Additionally, women taking OC were reported to experience an increase of 0.3°C in T_C, with HR observed to increase by 8 bpm^{-1} in response to exercising in a HH environment (30°C; r.h. 50%) [2]. Which could be indicative of a higher demand on $\dot{Q}$ to distribute blood to the skin for cooling in response to the elevated T_C, while maintain adequate blood flow to working muscles to meet the metabolic demands of exercise. Considering the association between high T_C and an increased risk of premature fatigue [361, 364], the role of OC on T_C should be investigated, to identify if exogenous progesterone and estrogen administration is causing further increases in T_C, ultimately increasing a woman's risk of premature fatigue.

2. 7. 5 Progesterone and the Cardiovascular System

Progesterone in its endogenous form has been shown [76] to increase minute ventilation throughout the luteal phase of the menstrual cycle and could be indicative of elevated cutaneous vasodilation to meet thermo-regulatory demands due to the elevated T_C associated with high levels of progesterone [390]. Similarly, several small studies [380, 385, 391-393] have identified higher HR at rest and throughout exercise during the luteal phase of the menstrual cycle, further substantiating the likelihood of a greater cardiovascular demand throughout periods of high progesterone secretion. Even though several studies [65, 68, 69, 394] concluded no substantial differences in HR at rest and in response to exercise in women throughout the menstrual cycle in TN and HH conditions [73, 74, 395, 396], increases in $\dot{Q}$, plasma volume, stroke volume and systolic blood pressure have been observed in women using monophasic OC for two-to-six months [397-399], which suggests that exogenous progesterone may affect the cardiovascular system in a similar manner to endogenous progesterone. Endogenous progesterone fluctuates throughout the various phases of the menstrual cycle, where as in monophasic OC, a constant supply is secreted throughout the 21-day active pill phase. Consequently women taking OC would experience these cardiovascular changes on a more consistent basis than women who are normally menstruating, which may influence exercise performance.

2. 7. 6 Thermoregulation

Thermoregulation is comprised of two mechanisms, namely heat dissipation and heat generation, which act to either cool down or warm up the body depending on the internal and external stimuli [400]. There are several mechanisms for heat dissipation within the human thermoregulatory system, namely radiative heat, convective heat and evaporative cooling [401]. Radiative heat involves the transfer of heat into the surrounding environment, via electromagnetic waves [401]. Convective heat involves the transfer of heat from one place to another via a fluid moving over a solid surface, displacing and replacing the fluid in immediate contact with the solid [401]. Both radiative and convective heat are used to maintain homeostasis throughout activities of daily living, however throughout exercise, these two methods of heat dissipation are often insufficient to attenuate rises in T_C at which point evaporative cooling becomes crucial. Evaporative cooling is a result of vaporisation of water from the skin (sweating), and the subsequent transfer of water vapour to the external environment [401]. Importantly, for effective evaporative cooling, the external environment must allow sufficient sweat evaporation, that is the environmental temperature should not exceed skin temperature [401], with relative humidity (r.h.) also playing

an integral role. The ability of active women to employ effective cooling mechanisms i.e. evaporative cooling, throughout exercise is crucial to avoid overheating. Endogenous progesterone increases T_C and the $T_{C\ THLD}$ for cutaneous vasodilation, thereby potentially delaying the onset of evaporative cooling, and considering exogenous progesterone in OC is secreted in higher concentrations than throughout the menstrual cycle, investigations must be undertaken to examine how exogenous progesterone is affecting T_C and consequently exercise performance, particularly in hot conditions when heat dissipation is crucial to avoid overheating.

2. 7. 7 Endogenous Ovarian Hormones and Thermoregulation

The cells of the endothelium [402] as well as smooth muscle in peripheral vasculature, [403] exhibit estrogen receptors which may induce vasodilation [404-406] or vasoconstriction in response to fluctuating hormone levels throughout the menstrual cycle, subsequently influencing blood flow [14, 407]. Additionally, estrogen and progesterone may influence sex-steroid binding neurons on the preoptic/anterior hypothalamus (PO/AH) [383, 408]. Progesterone reportedly reduces warm-sensitive neuron activity [47, 386, 388] subsequently, inhibiting the heat-loss mechanism and leading to increases in T_C. While estrogen inhibits coldand stimulates warm-sensitive neurones, which ultimately inhibits heat-retaining mechanisms, subsequently decreasing core body temperatures [14, 15, 383, 384]. When progestin alone was administered to women, elevations in T_C occurred, however when estrogen was administered alongside progestin, these changes in thermoregulation were not observed [14], suggesting that estrogen's effect on progesterone could attenuate changes in T_C. Further research demonstrated that while estrogen may attenuate some of the effects of progesterone, when both hormones are consistently elevated i.e. in the luteal phase of the menstrual cycle, T_C remains increased, suggesting progesterone has more pronounced effect on thermoregulation than estrogen [380, 385-387]. Progesterone has therefore been closely associated [12-15] with T_C. If endogenous progesterone is playing a role in determining the T_C response at rest and in response to exercise, it is possible that women taking OC, who are consistently exposed to progesterone in higher amounts, may experience further increases in T_C which may affect the initiation of thermoregulatory mechanisms throughout exercise.

Increases in T_C have been associated with elevations in the threshold for cutaneous vasodilation throughout exercise [380, 385, 392, 409, 410] during the luteal phase of the menstrual cycle. In normally menstruating women, differences in the $T_{C\ THLD}$ have been observed [380, 385, 410, 411]

between the follicular and luteal phases of the menstrual cycle, further demonstrating the link between elevated progesterone levels and a higher $T_{C\ THLD}$ [412, 413]. A higher threshold throughout the mid-luteal phase has been observed [413, 414] compared to the mid-follicular phase of the menstrual cycle, when progesterone levels were elevated and decreased respectively. Similar findings have been reported in exercising women, with increases in oesophageal temperature and the threshold for phalangeal cutaneous vasodilation observed [380, 385, 409-411] to be higher throughout the mid-luteal phase of the menstrual cycle in response to passive changes in ambient temperature. Additionally, exercising women with an oesophageal temperature higher than 37.5°C were observed [392] to have a 40-50% increase in forearm skin blood flow throughout the luteal phase when compared to the follicular phase of the menstrual cycle, after the $T_{C\ THLD}$ was reached. This may be an over-compensatory mechanism as the T_C may have been required to reach a higher point before cooling mechanisms were initiated, potentially placing the exercising woman under more stress than if cooling was initiated earlier, which may increase $\dot{Q}$ demands to maintain the metabolic loads associated with exercise and could have substantial implications for metabolic capacity while exercising [392]. The increase in $\dot{Q}$ could explain the elevated HR response observed [76] in women exercising throughout the luteal phase of the menstrual cycle, and could place the exercising woman in a greater state of stress throughout the luteal phase of the menstrual cycle, when T_C is elevated and thermoregulation is less efficiently achieved, which may have implications for the health and exercise performance of the woman.

2. 7. 8 Exogenous Ovarian Hormones and Thermoregulation

While little is known about the role of exogenous ovarian hormones in thermoregulation, considering the effect of the menstrual cycle on central regulatory mechanisms [390, 415-417], it is possible that exogenous estrogen and progesterone would cause similar changes to T_C and the $T_{C\ THLD}$ for cutaneous vasodilation. The increase in T_C observed [390, 400] throughout the luteal phase of the menstrual cycle may also occur during high-dose progesterone phases of some COC or while using progesterone-only pills. Increases to the $T_{C\ THLD}$ in monophasic OC users have been reported [390, 415], which may be due to the higher level of exogenous progesterone secreted in COC when compared with endogenous secretions throughout the normal menstrual cycle [108]. However, the change in basal body temperature from 0.3°C to 0.5°C observed throughout the luteal phase of the menstrual cycle [76, 382] is reportedly less pronounced in OC users [43]. This may reflect a consistent secretion of exogenous estrogen throughout the OC cycle compared with endogenous decreases following the rupture of the corpus lutem in the early stages

of the luteal phase. The constant secretion of estrogen may attenuate the effect of progesterone on T_C, and consequently women using COC with high estrogen doses may be less affected by progesterone-related increases in T_C and the $T_{C\ THLD}$ for cutaneous vasodilation. Nevertheless, the ability to minimise increases in T_C in active women may have substantial consequences for performance and the subsequent stress responses to exercise, and it is important to investigate the effect of exogenous estrogen and progesterone on T_C.

2. 8 SALIVA ANALYSIS IN RESEARCH

2. 8. 1 Saliva Composition

Saliva is a hypotonic fluid which plays a crucial role in health. Saliva has a pH of approximately 6.64 and a density between 1002 and 1012g/L [418, 419] and is comprised of numerous inorganic and organic compounds including electrolytes, water and proteins [420-422]. The predominant proteins in saliva include mucins, proline-rich proteins, lysosomes, peroxidases, lacoferrin, histatins, agglutinins, amylase, albumin and immunoglobulins [423]. Saliva provides a crucial antiviral and antibacterial role in the oral cavity [321] through its functions against pathogens [424], including defence proteins which act in either specific or non-specific manner, inhibiting the growth and replication of microorganisms [425, 426]. Approximately 20% of total plasma volume, which equates to around 750ml is translocated daily via the salivary glands [256, 427]. A secretion of between 750 to 1500 ml per day is considered healthy in humans [256, 419, 428], with an unstimulated saliva secretion rate of approximately 0.03-0.65 ml/min, and stimulated saliva as a result of mastication, secreted at a rate of 1.5-6.0 ml/min [428-430].

2. 8. 2 Saliva Analysis in Applied Sport Science Research

Saliva analysis is a valid, reliable and convenient way to measure several biomarkers of stress and health and is commonly used in applied sport science research [424, 431-433]. Saliva collection can be performed frequently, more rapidly and requires less medical training then blood collection [256, 418, 419, 434, 435] and can be readily used for diagnostic and health surveillance purposes [319, 320, 436, 437] in applied sport science research [435]. In addition, very low levels of a specific biomarker and low abundant proteins can be detected in saliva samples [436, 438, 439], potentially decreasing the risk of non-specific interference and hydrostatic interactions [440]. A sample volume of between 0.5ml-2.0ml is generally sufficient to elicit an accurate saliva analysis, depending on the sensitivity and reliability of the analysis system being utilised [441]. Saliva analysis is the preferred method of evaluation in younger individuals as it can be difficult,

undesirable or unethical to rely on blood and urine collection [332]. Additionally, saliva has been described as a filtration of blood, in that it reflects the physiological condition of the body [440, 442]. Saliva is not as complex and varying as serum, which has been shown to exhibit substantial variations in protein concentrations, with protein half-lives ranging from a few seconds to several months [440]. Despite reports of the total concentrations of the various compounds found in saliva being less than their respective serum and plasma concentrations; they have been reported to provide a reliable reference for analysis [443, 444], and could be a useful marker of monitoring mucosal immunity in athletes [445]. The systemic circulation and the salivary ducts are separated by a thin layer of epithelial cells, thus circulation facilitates the transfer of substances to the saliva through either active carriage, diffusion through the cell membrane or via a concentration gradient [440]. The lenience of this exchange is a major reason why saliva may be ideal for diagnostic purposes in an applied sport science environment. The serum components of saliva are derived from the vasculature which originates from the ceratoid arteries [446] and contains the same molecules found in systematic circulation [420]. Oral fluid analysis has been suggested to be more advantageous over serum samples due to better performance and efficiency than plasma analysis [424]. Additionally, some salivary measures, namely steroids, have been suggested to provide a more sensitive marker of changes in hormones than blood [447-449]. Literature indicates [448] that salivary measures of IgA and cortisol are both valid and reliable in identifying immunosuppression, with saliva collection often used in applied sport science research due to its simplicity and non-invasive nature [256, 418, 419, 434, 435] in contrast to blood samples.

Saliva flow rate, concentration and volume have been shown to directly relate to the method of saliva collection [450, 451]. The methods include draining, spitting, suction and swabbing [451-453], for the collection of either unstimulated saliva or stimulated saliva. Approximately 40 mg of unstimulated salivary IgA/kg body weight is transported to the mucosal surface every day [272]. Saliva can be stimulated via mechanical stimulation e.g. chewing on paraffin or acid stimulation[454, 455]. The usual method for saliva stimulation is mastication (chewing on paraffin) or gustatory stimulation (i.e. use of citric acid or sour candy drops on the participants tongue). However these foreign substances used to stimulate saliva tend to affect the fluid pH level and can stimulate the water phase of saliva secretion, subsequently causing a dilution in the concentration of proteins in the sample [456, 457]. To avoid such disturbances to results, unstimulated saliva was utilised throughout the present thesis. Whole saliva can be obtained with or without stimulation, however major differences in salivary proteins can be obtained if saliva is

stimulated [420]. Unstimulated saliva can have two components, namely spontaneous secretion and reflex secretion [288]. Spontaneous secretion refers to low amounts of saliva being continuously produced without an extraneous stimuli [288]. Reflex secretion involves minor stimulation through drying of the oral mucosa or mild mechanical stimulation e.g., minor jaw and tongue movements or mastication [288]. Stimulated saliva consists mainly of water and is derived from the parotid and submandibular salivary glands [288, 458]. Whereas a large portion of unstimulated saliva is derived from the submandibular salivary gland, with significant contributions from the sublingual and minor salivary glands [288] and contains proline rich proteins [439] and approximately three times more IgA than stimulated parotoid secretion [459, 460]. This has been replicated in whole saliva with similar results [461]. Even though it is possible to attain isolated samples from specific salivary glands, whole saliva is the only practical collection method for field-based research[233].

2. 8. 3 The Secretion of Saliva into the Oral Cavity

Whole saliva is comprised of a variety of oral fluids secreted in different volumes from three major and 600 minor salivary glands [288, 420, 430]. The major salivary glands include the submandibular, parotid and sublingual which contribute 65%, 23% and 4% of saliva into the oral cavity respectively [420], with minor salivary glands contributing 8% of saliva into the oral cavity [420]. Each salivary gland has an abundance of blood vessels and nerves which connect to each gland at the hilum and gradually separate into branches [435]. Arterial capillaries supply the parotid glands and originate from the external carotid artery, the sublingual and submental arteries supply the sublingual glands and the facial and lingual arteries supply the submandibular glands [256]. The salivary glands are comprised of several excretory units (acini) and a distinctive duct system [292]. Primary glandular (i.e. acinar) cells assemble to form a sac-like lumen, which forms the acini [292]. The acinar cells are categorised as either mucous or serous cells, depending on which type of cell they secrete [292], with each duct secreting a distinctive set of proteins [292] and each saliva gland producing a combination of secretions of several groups of cells which combine to constitute whole saliva [292]. The frequency at which s-IgA is secreted into the oral cavity is determined by plasma cells producing IgA in the submucosa and/or the rate of IgA transcytosis across epithelial cells, which relies on the availability of the pIgR [269]. It is possible that only intense exercise is sufficient to induce increased mobilisation of the pIgR [462], which could be the reason why moderate intensity exercise does not elicit substantial changes in s-IgA secretion rate and concentration [463]. The fluids secreted into the oral cavity from the major and

minor salivary glands contain epithelial immune cells, microbes and serum transudate from the mucosa and sites of inflammation [464, 465]. A number of factors can affect saliva composition, including the source of saliva (whole saliva or individual glands), flow rate, nature of the stimulus or physical exertion, diurnal and hormonal variations and the degree of hydration of the subject [256, 321, 430, 466, 467].

2. 8. 4 Saliva Gland Innervation and Saliva Secretion

Salivary glands are innervated by both parasympathetic and sympathetic nerves [435], which can influence saliva secretion and flow rate through the vasoconstriction or vasodilation of the blood vessels supplying the salivary glands [256, 468, 469]. While standard whole saliva secretion and composition is predominantly regulated by the autonomic nervous system [256, 470], increases in saliva secretion are reportedly due to parasympathetic nervous system stimulation leading to the vasodilation of the capillaries which supply the saliva glands, resulting increased blood flow [471]. Whereas decreases in saliva flow rate have been identified as a result of vasoconstriction of the blood vessels surrounding the oral mucosa [256, 472] due to sympathetic nervous system activation in response to intense and prolonged exercise [473].

2. 9 SUMMARY

The ability for a woman to tolerate and recover from exercise is crucial for maintaining health as well as improving performance. Several components of physiology including the mucosal immune system and the cortisol-driven stress response are known to be influenced by the frequency, duration and intensity of exercise [308, 474-477]. It is important that women are aware of how these parameters of physiology are affected by taking OC. The normal menstrual cycle can affect resting concentrations of s-IgA and sCort, which may also pose implications during and following exercise, with external factors such as ambient temperature providing additional complications, particularly in relation to maintaining homeostasis by regulating T_C. Considering the relative concentrations of endogenous and exogenous estrogen and progesterone secreted throughout the normal menstrual and OC cycles vary, both must be considered separately to provide novel insight into a woman's ability to tolerate exercise in both TN and HH environmental conditions. The aim of the present thesis is to explore the effect of exogenous estrogen and progesterone on the [s-IgA] and [sCort] response to exercise in varying environmental conditions. The insight gained from this investigation may assist in exercise prescription and recovery in

active women to reduce the risk of mucosal immune suppression and/or rising cortisol levels, which may compromise health.

CHAPTER THREE

Experimental Study One

The Validity and Reliability for a Salivary Cortisol Point of Care Test

3. 1 Introduction

Saliva has previously been described as a filtration of blood, in that it reflects the physiological condition of the body [440, 442]. The systemic circulation and salivary ducts are separated by a thin layer of epithelial cells, thus circulation facilitates the transfer of substances in to saliva through either active carriage, diffusion through the cell membrane or via a concentration gradient [440]. The lenience of this exchange is a major reason why saliva may be ideal for diagnostic purposes in an applied sport science environment, in which venepuncture may be inconvenient or unsanitary. Cortisol is amongst the most commonly assayed biomarker in saliva and is considered to be a true reflection of serum and plasma levels in healthy adults [424, 431-433] newborns [478], children and adolescents [479] and elderly subjects [480]. Salivary cortisol remains consistent with serum cortisol levels at rest and throughout exercise as well as upon stimulation of adrenocorticotropic and corticotropin-releasing hormones [449, 481, 482]. Very low levels of a specific biomarker and low abundant proteins can be detected in saliva samples [436, 438, 439], and therefore the risk of non-specific interference and hydrostatic interactions is potentially decreased [440]. Additionally, a sample volume of between 0.5ml-2.0ml is generally sufficient to elicit an accurate saliva analysis, depending on the sensitivity and reliability of the analysis system being utilised [441], and can be performed frequently, more rapidly and requires less medical training then blood collection [256, 418, 419, 434, 435] and therefore presenting a more attractive and athlete-friendly option for use in applied sport science research and practice [319, 320, 435-437, 445].

Cortisol is secreted in response to intense exercise [483, 484], via HPA axis activation [435]. Cortisol is primarily catabolic in nature and when secreted causes a reduction in protein synthesis and the inflammatory process, while increasingly protein degradation and compromising several parameters of immune function [182, 183]. Monitoring cortisol levels may provide the coach or sport scientist with valuable information regarding the stress response to exercise or training [435], which may assist in monitoring athlete well-being as well as adaptation to-and recovery from exercise. Over a sustained period of time high exercise-induced salivary cortisol levels may reflect overtraining [485], which can lead to a reduction in peak power, as well as in isometric and

concentric muscle contractions [486]. When symptoms of overtraining are appearing in an athlete, early diagnostic measures, rest and regeneration are key to recovery [487]. The availability of non-invasive and convenient measures to remotely monitor the health and well-being of an athlete may allow coaches and sport scientists to monitor the exercise-induced stress response throughout training and may be used in conjunction with medical diagnosis, for the early detection of over-training.

Traditional salivary cortisol tests can take several hours to receive a result. When investigating the cortisol response to exercise, timely feedback is crucial for the appropriate management of the athlete or active individual. An innovative point of care system for salivary diagnostics has been developed for cortisol monitoring and can provide feedback within 15 min of sample collection. This point of care saliva analysis system is known as IPRO and has the potential to drastically change the way coaches and athletes can monitor the stress response to training and competition. The IPRO point of care salivary cortisol test has undergone preliminary validity and reliability investigations in English Football League Championship Academy soccer players [488]. Twenty-nine saliva samples was measured by the IPRO and was also analysed by ELISA within 4 hr of sample collection. All samples were reported [488] to be within the measurement range for both IPRO and ELISA analysis ranging between 1.09 -9.45 ng/ml and 0.50 – 6.26 ng/ml respectively. In addition, 16 IPRO samples were measured on two separate LFD on two occasions, each 30 min apart, reporting [488] good agreement with mean within coefficient of variation (CV) between repeats of 6.88%. Similar results have been produced for a point of care test on s-IgA for the IPRO system [488-490]. The day-to-day reliability of the IPRO device has not been investigated which would establish the suitability of the device for ongoing monitoring of salivary cortisol.

The aim of the present study is to investigate the validity and reliability of the IPRO point of care sCort test against gold-standard ELISA methods in healthy, active individuals.

3. 2 Methods

3. 2. 1 Participants: Ten (n = 10, male = 5 and female = 5) healthy, active university students volunteered to participate in the present study. Participants were Exercise and Sport Science students from the same cohort, who participated in weekly recreational exercise such as running, cycling, swimming and team sports. Participants were instructed to refrain from strenuous

exercise 12 hr prior to saliva collection on both testing occasions. Participants were also instructed to avoid consuming food or brushing their teeth in the two hr prior to sample collection and had not consumed alcohol for at least 24 hr. Participants were asked to keep their morning routines as similar as possible on each day of sample collection, with testing occurring at the same time and location on both testing occasions. All participants signed informed consent prior to participating in this study. This study was approved by the Bond University Human Research Ethics Committee (BUHREC).

3. 2. 2 Sample Collection: Unstimulated whole saliva was collected by IPRO OFC (IPRO Interactive, Wallingford, UK), Salimetrics SOS (Salimetrics LLC, State College, PA, USA) and Salimetrics PD (Salimetrics LLC, State College, PA, USA) and assessed for salivary cortisol concentration ([sCort]). Participants were seated in a quiet, safe environment for saliva collection and asked to rinse their mouth out with water 10 min prior to sample collection. Saliva collection occurred on the same day and time, on two occasions separated by four weeks. Participants were considered to be under the same physiological and psychological stress surrounding both testing occasions. However, it is important to note that perceived physical and psychological stress was not measured through perceived stress scales which take into consideration recent life events, chronic difficulties, trait anger and depression [491]; all factors which may influence cortisol secretion at rest, and poses a limitation of the present study. During trial one (T1), a total of three saliva samples were collected (i.e. one of each OFC, SOS and PD, in that order) (Figure 7). During the second trial (T2) the same measures were collected, in order from T1, however two OFC's were placed in the oral cavity simultaneously (OFC_1 and OFC_2) in four randomly selected participants (Figure 7). The additional OFC samples collected in T2 were analysed on two separate lateral flow devices (LFD_1 and LFD_2) (Figure 7) and compared to investigate the reliability of both the OFC swab as well as the LFD using the IPRO method.

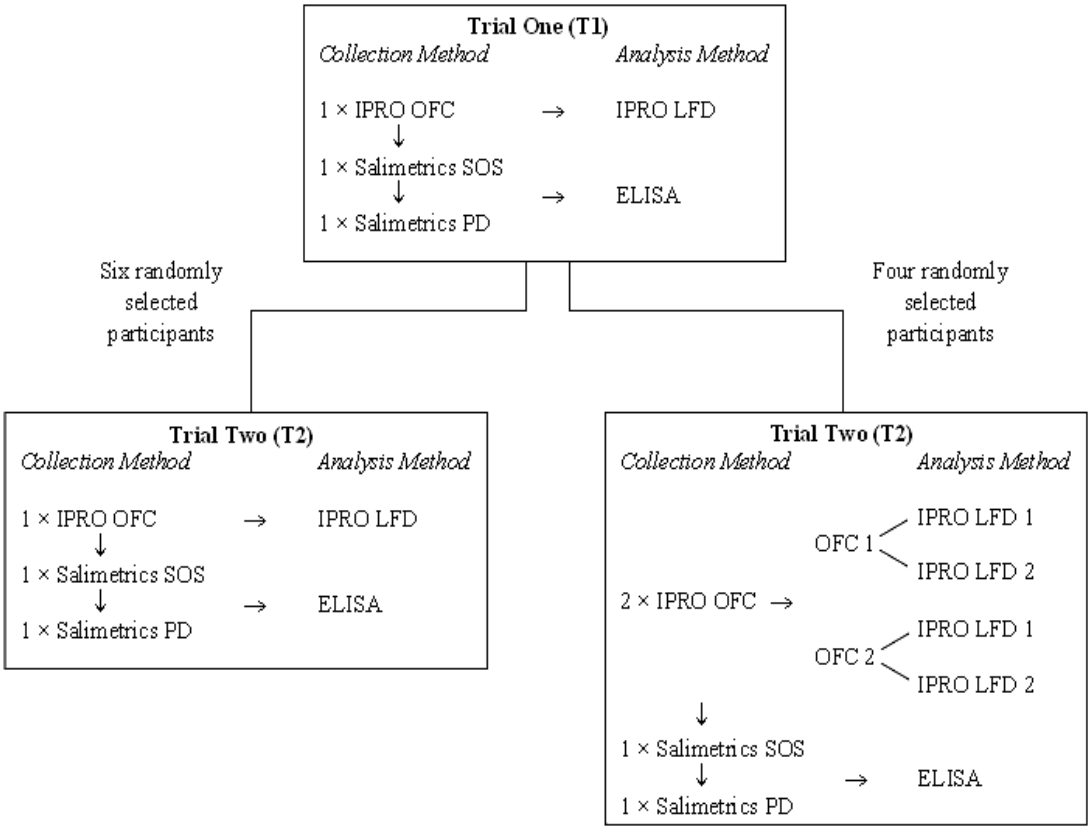

Figure 1. Schematic of sample collection procedures in trial one (T1) and trial two (T2).

3.2.3 Salivary collection and analysis procedures: Oral fluid collector (OFC): Participants placed one OFC swab on top of their tongue and closed their mouth in T1 with two OFC swabs placed on the tongue in T2 (in four randomly selected participants only). The device consists of a synthetic polymer-based swab material attached to a volume adequacy indicator stem and a dropper bottle with extraction buffer (Figure 9A). When 0.5mL of saliva has been absorbed by the OFC, an indicator line of the swab stem turns bright blue at which point the OFC is removed from the participant's mouth and placed (swab down) into a dropper bottle (Figure 9B). The dropper bottle contains 3mL of buffer which consists of sodium phosphate, salts, detergents and preservatives to attenuate the growth of microorganisms, as well as extraction agents to target analytes from the cotton based swab. Saliva samples are stable at room temperature and were stored according to manufacturer's recommendations (IPRO Interactive, Wallingford, UK).

For analysis, the dropper bottle containing 3mL of buffer and the OFC saliva sample was repeatedly inverted with care lightly shaken for 2 min. Two drops of the buffer solution and saliva

sample mixture was placed onto the sample pad of the LFD (Figure 10C) and incubated at room temperature for 10 min, as per manufacturer's instructions (IPRO Interactive, Wallingford, UK). The liquid uses a capillary action to travel through the conjugate pad hydrating the dried conjugate. The liquid continues to flow through the nitrocellulose membrane towards the wicking pad at the end of the strip. Once the liquid flows across the membrane, the gold-labelled anti-cortisol will be captured by the cortisol test line resulting in the appearance of a red line. Cortisol binds to the gold-labelled anti-cortisol molecule; and if cortisol is present, it will result in fewer gold particles being captured by the cortisol test line. The test line intensity is inversely proportional to the cortisol concentration present in the sample. After 10 min of incubation, the LFD was placed into the Lateral Flow Reader and analysed which then converted the line intensity into the corresponding [sCort].

Salimetrics oral swab (SOS): To standardise saliva collection procedures between point of care and ELISA testing methods, the SOS was used as it is similar to the OFC. The SOS is a polymer cylindrical swab (10mm × 30mm) (Figure 11A). Participants were instructed to place the SOS on top of their tongue and close their mouth, mimicking the OFC placement and procedure. After 2 min of saliva collection the swab was placed into storage tube (Figure 11B) and centrifuged at 1500 × g (3000 rpm) for 15 min as per manufacturers guidelines (Salimetrics LLC, State College, PA, USA). The saliva samples were weighed and then immediately stored in a freezer at -20°C until ELISA analysis.

Salimetrics passive drool (PD): PD was collected in 2ml cryovials (Figure 12A) (Salimetrics LLC, State College, PA, USA) and then sealed tightly. Saliva was passed through the SalivaBio collection aid into the polypropylene vial (Figure 13). Participants were encouraged to tilt their head forward throughout sample collection to promote saliva pooling in the oral cavity. Following collection all samples were centrifuged at 1500 × g (3000 rpm) for 15 min. The saliva samples were weighed and then immediately stored in a freezer at -20°C until ELISA analysis.

For analysis of SOS and PD, samples were thawed completely, placed on the vortex and centrifuged at 1500 × g (3000 rpm) for 15 min as per manufacturer's guidelines (Salimetrics LLC, State College, PA, USA). All saliva samples, reagents and the assay plate were at room temperature, prior to analysis. Wash buffer concentrate of 100mL was added to 900mL of deionised water at room temperature for dilution. Reagents were prepared and the assay plate

layout was determined, testing all samples in duplicate. Subsequently, 24 mL of diluted assay was then pipetted into the disposable tube, followed by 25 µL of assay diluent into two wells each to serve as the zero and non-specific binding (NSB) wells. A further 25 µL of standards and saliva samples were added into the appropriate wells. The enzyme conjugate was diluted 1:1600 by combining 15 µL of conjugate to the 24 mL tube of diluted assay, which was placed on the vortex prior to adding 200 µL to each well using a multichannel pipette. The assay plate was mixed on a plate rotator (SpectraMax 190, Molecular Devices, Fullerton CA, USA) for 5 min at 500 rpm and incubated at room temperature for 1 hr. Following incubation, the plate was washed four times with 1X buffer, with the plate thoroughly blotted on paper towels after each wash, prior to being turned upright. Following washing, 200 µL of tetramethylbenzidine (TMB) substrate solution was added to each well with a multichannel pipette. The assay plate was the mixed on a plate rotator or 5 min at 500 rpm and incubated in the dark at room temperature for 25 min. Following incubation, 50 µL of a 3M stop solution was added to each well with a multichannel pipette. The assay plate was mixed on a plate rotator for a further 3 min at 500 rpm, or until all wells turned yellow. The assay plate was then read in a plate reader at 450 nm, within 10 min of adding the 3M stop solution.

3. 2. 4 Statistical Analysis: Data was analysed for assumptions of normality using a Shapirio-Wilk test, and in the event data was violated non-parametric Friedman two-way ANOVA or Wilcoxon Signed Rank tests were conducted. A Paired t-test was completed to compare $IPRO_{T1}$ and $IPRO_{T2}$, as well as $IPRO_1$ and $IPRO_2$ and LFD_1 and LFD_2 samples for intra-tester and OFC and LFD reliability, with ICC ±SEM presented. Pearson's correlation coefficient and One-way Random model for intra-rater reliability were completed to confirm reliability. A Bland-Altman graph was used to assess the level of agreement between IPRO compared with SOS and PD with at 95% CI reported. Alpha intervals were set at $p < 0.05$ for all correlation and hypothesis tests with results presented as mean ±SD. Statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS version 20, Chicago, IL, USA).

3. 3 Results

3. 3. 1 Validity

A Friedman two-way ANOVA did not identify any significant ($p = 0.819$) difference between OFC and SOS or PD. A follow up analysis of pair wise comparisons was completed in the form of a Wilcoxon signed rank test, revealing OFC was not significantly different from SOS ($p =$

0.881, r = -0.33*)* or PD (p = 0.145, r = 0.32) measures. The level of agreement between IPRO vs SOS (Figure 8) and IPRO vs PD (Figure 9) were assessed via a Bland-Altman graph, revealing unbiased agreement between OFC and both SOS and PD methods. Validity and reliability statistics for OFC, SOS and PD are presented in Table 1.

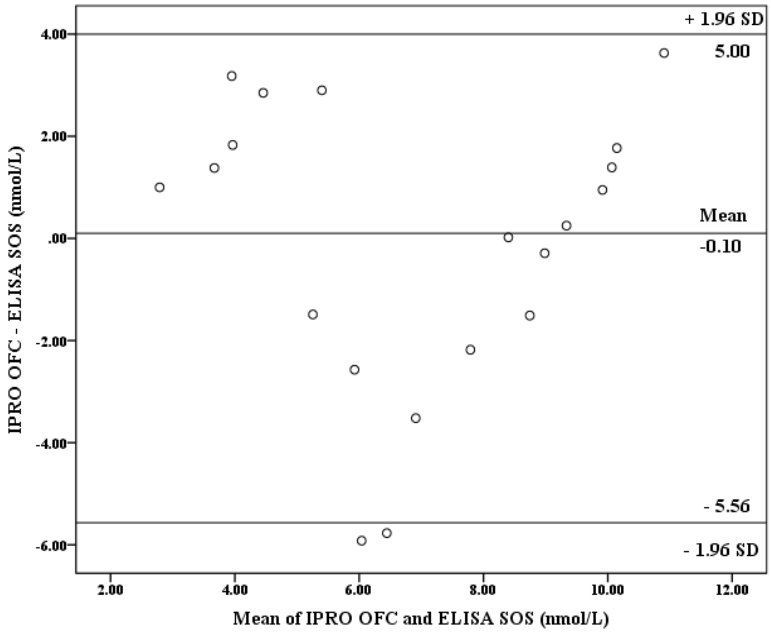

Figure 2. Bland-Altman scatter plot, agreement between Individual Profiling (IPRO) oral fluid collector (OFC) and ELISA Salimetrics oral swab (SOS). Solid lines indicate mean difference (-0.10 nmol/L), and ±1.96 SD (5.00 nmol/L, -5.56 nmol/L). No proportional bias was present (p = 0.976).

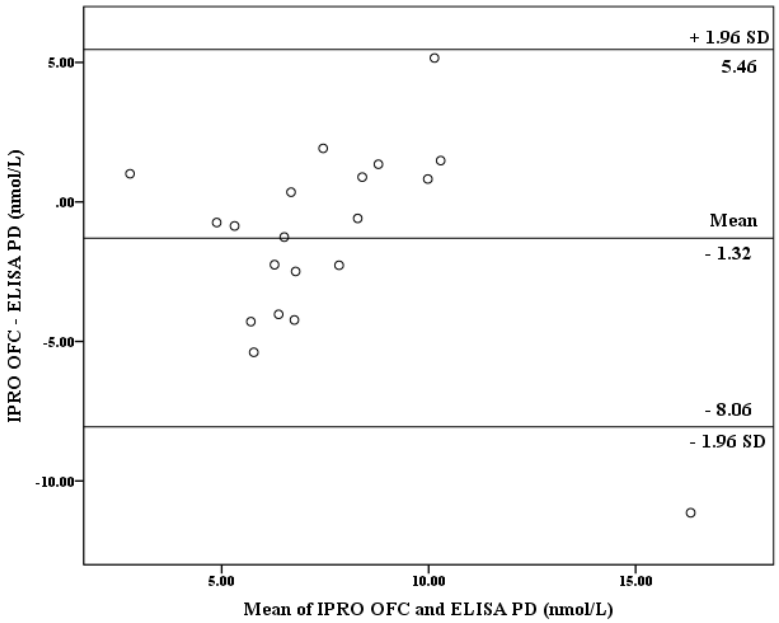

Figure 3. Bland-Altman scatter plot, agreement between Individual Profiling (IPRO) oral fluid collector (OFC) and ELISA passive drool (PD). Solid lines indicate mean difference (-1.32 nmol/L), and ±1.96 SD (5.46 nmol/L, -8.06 nmol/L). No proportional bias was present (p = 0.281). One outlier was identified.

3. 3. 2 Reliability

Tests of normality showed no violation and Paired t-tests were conducted to establish reliability. No significant differences (p = 0.223, ICC = 0.685; p = 0.02 ±0.7 SEM, d = 0.06) were observed between OFC_{T1} and OFC_{T2}, indicating good inter-tester reliability. Additionally, no significant difference was found between OFC_1 (6.26 ±2.84 nmol/L) and OFC_2 (6.46 ±3.38 nmol/L) or LFD_1 (6.35 ±3.01 nmol/L) and LFD_2 (6.37 ±3.00 nmol/L). Indicating good reliability of both the OFC swab (p = 0.813, ICC = 0.890; p = 0.11 ±0.4 SEM) and LFD (p = 0.977, ICC = 0.850; p = 0.00 ±0.5 SEM, d = 0.00). Descriptive statistics for OFC, SOS and PD are shown in Table 2.

Table 1. Validity and reliability statistics for Individual Profiling (IPRO) oral fluid collector (OFC), ELISA Salimetrics oral swab (SOS) and ELISA Salimetrics passive drool (PD) for salivary cortisol concentration [sCort].

	Value	95% upper limit	95% lower limit
Validity			
IPRO OFC vs. ELISA SOS			
[sCort] Mean Difference (nmol/L)	-0.10 ±2.79	1.20	-1.41
Pearson's Correlation Coefficient (*r*)	0.52		
IPRO OFC vs. ELISA PD			
[sCort] Mean Difference (nmol/L)	-1.32 ±3.45	0.28	-2.94
Pearson's Correlation Coefficient (*r*)	0.45		
Reliability			
IPRO OFC_{T1} vs. OFC_{T2}			
[sCort] Mean Difference (nmol/L)	-0.93 ±2.26	0.68	-2.55
Intra Class Correlation (ICC)	0.658	0.901	0.125
Pearson's Correlation Coefficient (*r*)	0.68		
IPRO OFC_1 vs. OFC_2			
[sCort] Mean Difference (nmol/L)	-0.20 ±1.55	-2.67	2.27
Intra Class Correlation (ICC)	0.904	0.993	0.331
Pearson's Correlation Coefficient (*r*)	0.89		
IPRO LFD_1 vs. LFD_2			
[sCort] Mean Difference (nmol/L)	-0.01 ±1.64	-1.39	1.35
Intra Class Correlation (ICC)	0.868	0.971	0.514
Pearson's Correlation Coefficient (*r*)	0.85		

Table 2. Descriptive statistics for Individual Profiling (IPRO) oral fluid collector (OFC), ELISA Salimetrics oral swab (SOS) and ELISA Salimetrics passive drool (PD) for salivary cortisol concentration [sCort].

	OFC	**SOS**	**PD**
[sCort] Mean ±SD (nmol/L)	6.90 ±2.88	7.00 ±2.86	8.23 ±3.63
[sCort] Mean 95% CI - Lower	5.55	5.66	6.53
[sCort] Mean 95% CI - Upper	8.25	8.34	9.93
[sCort] Minimum	3.08	2.29	2.28
[sCort] Maximum	12.72	9.50	21.90

3. 4 Discussion

The results of the present study found the IPRO point of care test for salivary cortisol concentration [sCort] to have good agreement with the industry gold standard ELISA method. Additionally, the present study identified the OFC method of salivary collection to be more closely correlated with the SOS method ($p = 0.881$; $d = 0.06$) compared with PD method ($p = 0.145$; $d = 0.00$) for [sCort]. This is an important finding, and suggests future salivary cortisol comparisons should preferentially compare OFC results to SOS results when necessary. This finding is in-line with previous research [492] reporting differences in results in Salivary Immunoglobulin A concentrations in cotton swab collection compared with passive drool collection and demonstrates the importance of standardising saliva collection techniques in research and practice. Both OFC ($p = 0.813$) and LFD ($p = 0.977$) duplicate samples were not statistically different, indicating good reliability between samples. This finding suggests the IPRO point of care method is useful for salivary cortisol testing in recreationally active individuals and investigators can be satisfied that any substantial inter-subject changes to [sCort] are not due to tester errors if proper testing protocol is followed. The results of the present study are in agreement with previous investigations in English Football League Championship Academy soccer players [488] finding the IPRO method to be in good agreement with the ELISA method for determining [sCort]. Together, these findings suggest the IPRO method of measuring salivary cortisol (via OFC collection and LFD analysis) is useful for coaches and researchers when monitoring salivary cortisol in both recreationally active individuals and elite level athletes.

Saliva analysis is commonplace in applied sport science research due to convenience of sample collection with between 0.5-2.0ml generally sufficient to elicit an accurate result [319, 320, 435-437, 445], however sample analysis can be time consuming. The IPRO method produces results within 15 min of sample preparation, providing timely feedback, and therefore offering an appealing alternative method of stress monitoring in real-time. In addition, the IPRO LFD reader is portable and can be used in the field for real-time analysis, bridging the gap between laboratory and field testing in applied sport science research and practice. The immediate availability of information regarding physiological demands of exercise is crucial in athlete monitoring. Coaches using the IPRO method for [sCort] analysis can readily monitor the stress response of their athletes throughout training and competition, gaining specific feedback about the demands of sport and exercise, providing advantage over traditional salivary cortisol measures i.e. ELISA.

The results of the present study demonstrate the validity and reliability of a point of care salivary cortisol test for use in recreationally active individuals. Both the IPRO saliva collection (OFC) and analysis (LFD) aspects of the method were shown to be valid and reliable for salivary cortisol monitoring of [sCort] compared with the ELISA technique. Further reliability and validity testing should be conducted for the IPRO salivary cortisol method to confirm the findings of the present paper in a larger and more applied sample population including elite athletes.

3. 5 Practical Applications

The IPRO method showed a good agreement with the ELISA method for salivary cortisol analysis. In addition, the IPRO OFC and LFD show good reliability between samples and may prove to be a useful method for coaches and sport scientists to monitor changes in [sCort] in response to training and competition.

CHAPTER FOUR

Experimental Study Two

The Response of Salivary Immunoglobulin A to Elite Surf Lifesaving Competition

4. 1 Introduction

The influence of exercise on immune system function has been investigated in elite sports including swimming [313, 493] kayaking [306, 307] and running [305, 494]. In particular, URTI have been identified [495] as the most common infection experienced by highly trained athletes and are frequently responsible for athlete absence from training and competition [331]. Pyne (1999) [496] concluded that acquiring an URTI during training or competition may lead to detrimental effects on the health and performance of elite athletes.

Tomasi and colleagues [497] reported that a predisposition to URTI often observed in athletes may be a result of a depressed immune response following intense exercise. In particular, decreases in s-IgA, the most abundant immunoglobulin at the mucosal surfaces of the upper respiratory tract, have been shown [270] to initiate up to 95% of all infections. Salivary immunoglobulin A represents the first line of defence against pathogens by preventing colonization and replication of viruses and bacteria on the mucosal surface of the upper respiratory tract [28]. It has been demonstrated [270, 313] that decreasing concentrations of s-IgA are associated with a predisposition to respiratory illness and can be a useful marker for respiratory infection risk [31]. Saliva analysis has become a widely accepted and utilised measure in applied research and team sport monitoring strategies [306, 451, 493] due to the non-invasive nature of sample collection and improved analysis methodologies for determining the s-IgA concentration [319]. The s-IgA concentration has been shown [477] to correlate more highly with URTI than serum antibodies or other immune parameters and as such provides a worthwhile measure for integrated athlete monitoring protocols in individual and team sports.

Multiple studies [306, 315, 319] have investigated the effects of endurance exercise similar to that undertaken by elite SLS athletes, reporting decreases in s-IgA concentration in response to intense kayaking [306, 307] (>60% $\dot{V}O_2$ max), a seven month swim training program [313] and marathon running [305]. However, other studies [493, 498, 499] have reported no change in s-

IgA concentration following intermittent and continuous exercise, including soccer and rugby competition and prolonged (>1.5 hr in duration) swimming. Increased s-IgA concentration following cycling [500] and basketball competition and training [501] have been reported to further demonstrate the acute impact of exercise on the immune system. Mackinnon [264] identified that the degree of s-IgA suppression is dependent on the intensity of the exercise. Currently, a discrepancy exists with respect to the acute s-IgA response to exercise and may be associated with methodological differences related to sample size and method of collection.

Despite current research [307, 331, 496] examining the immune response to intense training for specific sports, few studies [499, 501] have investigated the changes in s-IgA concentrations during competitive exercise performance and the acute response of s-IgA to elite SLS competition is unknown. Elite SLS competition is a physically demanding sport that requires substantial physical conditioning, with athletes regularly participating in multiple daily training sessions - six days a week. In SLS, the 'ironman' race has emerged as the most demanding multi-disciplinary event through the consecutive performance of the four major skills of SLS namely soft sand beach running, surf swimming, surf board paddling and surf ski paddling [502] in a single event. Multi-disciplinary surf athletes complete each skill over varying course formats and distances and are associated with performance durations from fifteen min to four and half hours [503] for any one event. It is also common for athletes to compete in heats, semi-finals and finals on a single day of competition [502]. Despite the professional status of the sport, currently few studies [502] have investigated the effects of training and competition on the elite SLS athlete and there is a lack of understanding regarding the endocrine and immunological response of elite SLS athletes to training and competition. The aim of the present study was to investigate the s-IgA response of elite men and women to a single, endurance SLS competitive event. The endurance SLS event under investigation involved continuous loops of a competitive course, which integrated surf swimming, surf board paddling and surf ski paddling in the open ocean. Male competitors raced for approximately 90 min and female athletes competed for approximately 60 min. No previous studies have examined the relationship between the exercise-related response of s-IgA and the predisposition of athletes to develop URTI in SLS athletes following competition.

4. 2 Methods

Approach to the Problem: Athletes provided daily unstimulated saliva samples in a non-fasted state over a period of six days at approximately from 54 hr pre-event to 61 hr post-event. All

saliva samples collected at training sessions occurred at approximately 0500 hours. Saliva samples collected on the day of event varied in response to the schedules time of competition; pre-event samples were collected prior to warm up between 0900 and 1100 hours, while post-event samples were collected between 1300 and 1500 hours, after cool down. The daily training and saliva collection schedule is outlined in Table 1. Data were examined for each subject at each saliva sample collection time point. Throughout the pre and post-event sample collection period, subjects participated in all scheduled training and recovery sessions (Table 3).

Table 3. The daily training and saliva collection schedule of SLS athletes throughout the six-day testing period (n = 25).

Test	**48hr Pre-Event**	**24hr Pre-Event**	**Pre-Event**	**30min Post-Event**	**24hr Post-Event**	**48hr Post-Event**	**72hr Post-Event**
Training Schedule	0500 Swim	0500 Swim	0900 – 11:00 Pre Event	1300-16:00 Post-Event	0900 Recovery	0500 Swim	0500 Swim
Saliva Test	15min Prior to Training	15min Prior to Training	15min Prior to Warm Up	After cool down / recovery.	15min Post-Awakening	15min Prior to Training	15min Prior to Training

4. 2. 1 Participants: A total of 25 athletes competing in the 2012/2013 Kellogg's Nutri-Grain Ironman and Telstra Ironwoman series were invited to participate in this study. Eighteen (n = 18) participants (male n = 10, female n = 8) volunteered to participate in this study (age: 24.4 y ± 7.5 yr). All athletes that volunteered to participate in the study provided written informed consent and completed medical history and physical activity readiness questionnaires (PARQ). The protocol associated with the investigation was approved by the Bond University Human Research Ethics Committee (BUHREC).

4. 2. 2 Procedures: The present study was undertaken during one of the 2012/2013 Kellogg's Nutri-Grain Ironman and Telstra Ironwoman events which was approximately 90 min in duration and included all four skills of Ironman and Ironwoman competition; soft sand beach running, surf

swimming, surf board paddling and surf ski paddling that constituted separate stages of a single competition race. Each lap was a total distance of 1993m with the surf board paddling, surf swim, surf ski and run stages set at 662m, 421m, 750m and 160m respectively. Each skill was completed in sequence and repeated four times.

Unstimulated saliva was collected via an Oral Fluid Collector (IPRO OFC, IPRO Interactive, Oxfordshire, UK) consisting of a synthetic polymer based material on a polypropylene tube. The OFC has a volume adequacy indicator, providing a clear colour change when 0.5mL (±20%) of passive saliva is collected. All subjects were instructed to swallow any saliva present, before placing the OFC on their tongue. All subjects were requested to avoid the ingestion of food and fluids other than water in the 60 min before providing each saliva sample and to refrain from brushing their teeth two hours before each saliva sample collection session. Subjects were instructed to wait for a period of 10 min after their last consumption of water before commencing the saliva sample collection process. Saliva samples were collected from each athlete at their training and race venue as determined by the sample collection protocol to minimise disruption to the participants' pre-event routine and preparation.

Saliva Immunoglobulin A (µg/mL) was analysed in duplicate via a commercially available lateral flow immunoassay test kits (LFD; IPRO Interactive Ltd., Oxfordshire, England) and are reported as absolute measures for within individual variation. The lateral flow principle of saliva analysis, or immunochromatographic strip (ICS) test was conducted in accordance with manufacturer instructions. For the LFD, two drops of saliva/buffer mix are added to the sample window of the LFD cassette. The liquid runs the length of the test strip via capillary action creating a control and test line visible in the test window. Five min after the sample is added the test line intensity is measured in an IPRO plate reader (IPRO Interactive Ltd., Oxfordshire, England). The test line intensity is inversely proportional to the s-IgA concentration in the sample. The s-IgA intra-assay coefficient of variation as a percentage was 8.0%.

4. 2. 3 Statistical Analysis: All data are expressed as mean ±SEM. A one-way repeated measures analysis of variance (ANOVA) was used to compare differences in salivary IgA concentration for each saliva sample collection time point. When the ANOVA showed significant main effects, a Tukey's *post-hoc* test was used to identify the source of the differences. The statistical software package SPSS version 21.0 was used for all data analysis.

4. 3 Results

The mean baseline measures collected at 54 hr, 30 hr prior to and the morning of the event were 33.5 ±5.6 µg/mL, 38.6 ±8.8 µg/mL and 37.4 ±8.7 µg/mL respectively for males, and 31.0 ±6.2 µg/mL, 39.7 ±8.4 µg/mL and 34.0 ±7.2 µg/mL respectively, for females. A non-significant increase in s-IgA concentration was observed for both genders, post-event compared to pre-event with a measure of 44.3 ±8.4 µg/mL for males and 38.9 ±9.3 µg/mL for females. Samples collected 17 hr and 37 hr post-event were measured at 39.7 ±8.9 µg/mL and 39.8 ±7.9 µg/mL respectively for males and 32.4 ±6.7 µg/mL and 39.88 ±8.6 µg/mL respectively for females. No significant change was observed for females 61 hr post-event with a mean measure of 35.6 ±7.5 µg/mL, however a significant increase ($p < 0.05$; $d = 1.66$) in s-IgA concentration was observed at 61 hr post-event in males, compared to all other time-points, with a mean measure of 49.2 ±8.8 µg/mL. The mean values ±SEM for both genders are shown in Figure 4.

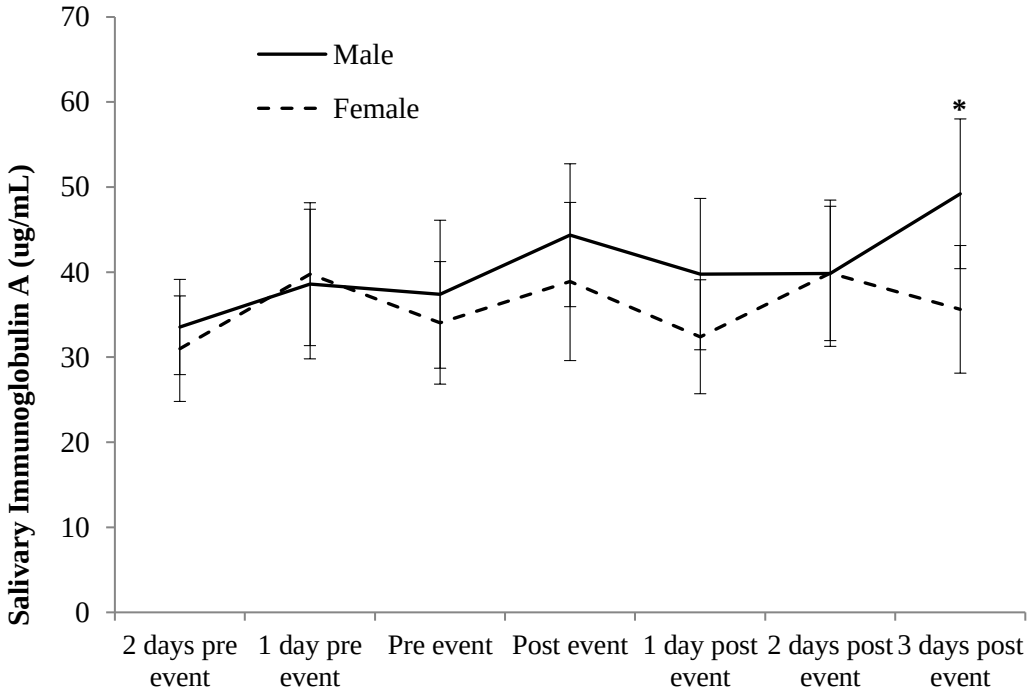

Figure 4. Changes in salivary IgA concentration before and after a single SLS event. Values are means ±SEM. * Significantly ($p < 0.05$) higher than all other male time-points.

4. 4 Discussion

The results of the present study provide new insight into the s-IgA response to elite SLS competition and demonstrate the unique characteristics of SLS in comparison to research [494]

that has reported a decrease in [s-IgA] in response to high intensity swimming [313] kayaking [306] and marathon running [305]. The results of the present study demonstrate that elite male and females SLS athletes experience a non-significant increase in [s-IgA] within 30 min post SLS endurance competition. Additionally, male athletes experience significant ($p < 0.05$; $d = 1.66$) increases in [s-IgA] 61 hr post-competition of approximately 90 min duration, when compared with all other time-points in males.

The mechanism responsible for the changes in [s-IgA] during the present study is unclear, however multiple studies [264, 504] have suggested a link between physical stress from intense exercise, life style factors and annual training mileage and fluctuations in [s-IgA]. A possible mechanism associated with the results of the present study is the influence of the stimulation of the HPA axis in response to intense exercise, which up-regulates the production of polymeric-IgA receptors and facilitates the active transport of the polymeric-IgA complex across epithelial cells [505] leading to increases in [s-IgA]. Upon stimulation, the nervous system has been shown to increase the secretion of s-IgA into saliva through the stimulation of the sympathetic and parasympathetic nervous systems [297, 298]. The autonomic nervous system supplies nerves to the immune cells in lymphoid organs, and has been shown to induce changes in local immune processing [299]. In particular, the autonomic nervous system has been shown to enhance the transfer of s-IgA [300]. The SLS event examined in the present study required athletes to physically exert themselves to exhaustion over approximately 90 min in attempt to be victorious and accumulate sufficient points in the series to guarantee automatic qualification for the subsequent competition. Combining the additional pressure of concerns over requalification for the following year with the challenging oceanic conditions of this particular single, endurance SLS event, it is therefore possible that physical exhaustion and psychological stress may have contributed to amplified HPA axis activation, subsequently increasing [s-IgA] as observed at 61 hr post-event in males, as an immune-compensatory effect.

Mackinnon [306] suggested intense daily kayaking (> 70% maximal HR) in elite athletes may result in adverse changes in the production and secretion of s-IgA. In particular, a malfunction of the mucosal plasma cells, which migrate from the gut to the minor salivary glands, which is the major source of IgA in whole saliva [306]. The adverse changes in the production and secretion of s-IgA may occur in response to variations in temperature in mucous membranes or from decreases in nasal fluids, ultimately leading to a reduction in IgA output [497]. Elite SLS athletes

undergo substantial physical conditioning to prepare for competition, with athletes commonly training for several years before achieving optimal strength and endurance to successfully compete at the elite level. It is possible that a cumulative effect of training history may have contributed to the changes observed in the present study.

Salivary IgA concentrations were not altered in male and female elite swimmers in response to a five month period of training leading into the 1998 Commonwealth games [493]. Conversely, intense training in a cohort of 26 elite swimmers over a five month period was directly linked to a suppression of mucosal immunity [315]. Similar results were observed over a three week training period in elite kayakers, showing decreases in [s-IgA] [306]. The distinction between the results of the present study and studies that examined swimming [315, 493] and kayaking [306] are likely to be associated with variation in training volumes, competitive distances, sample collection times and the unique nature of multi-event endurance sports such as SLS. The athletes in the present sudy were competing until exhaustion in a crucial event, it is therefore difficult to compare to other studies [315, 493] which investigated training sessions. It is possible that the same exercise intensity as experienced in a competition setting, was not obtained in these studies. Allgrove et al [506] investigated cycling to exhaustion, reporting very-high intensity exercise was sufficient to induce increased transportation of s-IgA into the saliva, immediately following exercise, as a result of sympathetic stimulation. Allgrove [506] suggested it is possible for exercise to exhaustion to activate the sympathetic nervous system (SNS) and cause an increase in [s-IgA]. Previous studies [507, 508] have concluded exercise induced changes in [s-IgA] return to resting levels within 24 hours of exercise cessation. It is possible that increased neural activation throughout the competition and upon the resumption of regular training at approximately 37-61 hr post-event, may have provoked SNS activation and subsequent increases in [s-IgA]. The increases in [s-IgA] observed post-event in both genders and 61 hr post-event in males may have been exacerbated by below normal baseline measures. It is possible that baseline measures were lower than average as particpants were in a taper period of reduced training volume in preparation for the event, and therefore the reductions in training volume may not have been sufficient to elicit a charcacteristic change in [s-IgA] in the days prior to the event.

The effect of gender on s-IgA has been investigated in the athletic population concluding females often exhibit slower whole saliva secretion rates than males [509] and consequently higher [s-IgA] than males [510]. The slower whole saliva secretion rate commonly observed in females

may be due to hormonal differences associated with gender or the use of OC [510]. The menstrual cycle and OC have been shown to influence the free cortisol response, with lower levels observed in the follicular phase of the menstrual cycle or while using OC compared with males [510]. Changes in the free cortisol response could lead to alterations in [s-IgA] as cortisol plays a role in the movement and functional capabilities of several immune cells including B-cells [511]. It has been reported [510] that gender-related secretion rates may be attenuated when male and females of the same age group are compared. The results of the present study exhibit similar results for males and females, with both genders showing non-significant increases in post-event means compared with pre-event means. Additionally both genders exhibit non-significant increases in [s-IgA] 17 hr post-event however only males were observed to have a signifanct increase in [s-IgA] 61 hr post-event. It is possible that male athletes returned to regular training following the event prior to female athletes and therefore the elevated [s-IgA] observed at 61 hr post-event for males could be attributed to increased neural activation associated with the resumption of high intensity training. Additionally, it is possible that differences in hormonal release from varying phases of the menstrual cycle or the use of OC could have reduced the [s-IgA] in females, however as no information was gathered regarding which phase of the menstrual cycle each female particpant was in at the time of sample collection, this remains anecdotal.

The present study observed absolute [s-IgA] in elite SLS athletes pre- and post-event, which is consistent with applied research methodologies that have investigated the impact of training on [s-IgA] and the relationship between [s-IgA] and competitive performance in swimmers leading into National Championships and the 1998 Commonwealth Games [315, 493]. Salivary IgA has been identified to be subjected to more short-term variations compared with other salivary proteins [456, 512] in addition to greater within- and between-subject variations in athletes such as elite rowers and swimmers [324]. Variation in the number of subjects, the demands of exercise, saliva collection and analysis methods reduces the ability for direct comparison between the results of the present study and others [307, 315, 493]. As a result of the high variability of s-IgA, studies with small sample sizes, such as the present study, may be less likely to detect real changes in salivary proteins and therefore any results should be interpreted with caution [513].

The present study found a significant increase ($p < 0.05$; $d = 1.66$) in [s-IgA] 61 hr post-event in males, compared with all other time-points. In addition, non-significant increases in [s-IgA] were observed post-event when compared to pre-event in both genders, which suggests increased SNS

activation associated with high intensity and prolonged exercise could have led to an increased secretion of s-IgA into the oral cavity. However, due to the applied population and resultant limited sample size available for participation in the present study as well as the high within- and between-subject variations in s-IgA it is difficult to draw unequivocal conclusions from the results of the present study. It is possible that a cumulative effect of substantial physical conditioning over several years has had an adverse effect on the mucosal immune system, resulting in abnormal variations in [s-IgA]. Further longitudinal research with a greater sample size using the IPRO device should be conducted to determine the immunological response to elite SLS Ironmen and Ironwomen competition and the validity and reliability of the device for use in an applied sport setting.

4. 5 Practical Applications

This study demonstrated the atypical response of elite surf lifesaving athletes to that previously reported for athletes of similar disciplines such as swimming and kayaking, highlighting the variability of immunosuppressive responses. The use of a point of care, field-based saliva analysis system may provide coaches and athletes with the ability to individually assess the immediate effects of training and competition on the immune system. Swift feedback on immune functioning following physical exertion could allow coaches and athletes to monitor immunosuppression, predict the risk of infection and adjust training periodization and recovery sessions accordingly. Elite SLS athletes and coaches may need to implement stringent precautions while tapering prior to major competitions, to reduce the risk of upper respiratory tract infection as a result of lowered [s-IgA] in response to reduced training load. The results of the present study indicate the s-IgA response in the days following SLS competition may vary between genders and therefore individualised recovery protocols should be implemented to minimise the risk of acquiring respiratory infections post-exercise and reduce the risk of resuming regular training prematurely.

CHAPTER FIVE

Experimental Study Three

Prevalence and the associated Benefits and Barriers for Oral Contraceptive use in Australian women grouped for physical activity level.

5. 1 Introduction

Oral contraceptives are one form of hormonal contraception that includes the administration of pills that vary in their type, amount and/or preparation of synthetic sex-hormones estradiol and progestin. An accurate account of the prevalence of OC use is difficult given the variability in research findings. For instance, 25-76% of Australian women reported using OC between 2001 and 2007 [160, 514, 515]. The wide range in the prevalence rate of OC use might be due to the variance in participant demographics particularly age, income, and education [160, 515, 516]. Of particular interest, the OC practices of female athletes were initially identified to be less prevalent when compared to the general population [514], although the prevalence of OC use specifically in Australian female athletes was reported to be similar to the general population in 2007 [160, 515]. Beals and Hill (2006) [123] demonstrated that the prevalence of OC use may be different among lean-build sports (e.g., cross-country) when compared to non-lean build sports (e.g., softball). Thus, in order to quantify and understand the rate and motive for OC use in active women, a range of sports and exercise levels should be included.

Despite the ambiguity surrounding the prevalence rates of OC among sample groups, it is clear that there has been increasing over the last three decades [134, 517, 518]. An increasing rate of prevalence could be attributable to the third-generation (3G) pills that contain lower dosages of estrogen and progestin resulting in fewer/reduced side effects such as weight gain, and nausea [519]. Despite the positive effect of 3G pills on side-effects, other benefits and barriers specific to sample groups in the population are an interesting consideration. It is reasonable to suggest that if the prevalence of OC differs among sports, then the perceived benefits and barriers to OC use might differ among sports.

The benefits and barriers for OC use in women have been explored in several survey-based studies [163-165] suggesting that cycle regularity, a reduction in dysmenorrhea, and acne relief were the most common known reasons for OC use, aside from birth control. Potential weight gain, nausea,

and mood changes such as depression, as well as an increased risk of some cancers and cardiovascular disease were reported as the predominant barriers associated specifically with OC use in American women [520]. Despite some information about the use of OC in the general population, less in known about the benefits and barriers to OC use specifically in active populations.

Recent research has identified an association between synthetic hormone administration and decrements in peak aerobic power [7, 8, 167] as well as a reduced ability to recover from eccentric exercise [521, 522]. These factors may constitute credible barriers for active women to avoid OC whereas they may be regarded as less important in less active women.

The purpose of this paper is to explore the 'benefits and barriers' for OC and specifically the role physical activity levels play on OC use in Australian women. It is hypothesized that the prevalence of OC use will be high in active compared to sedentary women, to allow for cycle regulation or manipulation. Furthermore, we expect that the non-contraceptive motives for OC use within less active women may be tailored towards attenuating symptoms associated with dysmenorrhea, treating acne, and cycle regulation.

5. 2 Methods

5. 2. 1 Participants: Participants (n = 125) were recruited via email and social media with the criteria for inclusion into their respective group based upon Exercise and Sport Science Australia (ESSA) physical-activity intensity guidelines [523] regarding recommended frequency, duration and intensity of physical activity.

Categorisation resulted in untrained women (UT; n = 26; 32.2 yr ±7.3 yr; i.e. < 2 sessions/wk of low intensity exercise or < 2 METS/session), recreationally active (REC; n = 44; 30.6 yr ±6.8 yr; i.e. 3-4 sessions/wk of moderate intensity exercise or 3-6 METS/session) and trained (TR; n = 55; 27.0 yr, ±8.3 yr; i.e. 5+ sessions/week of vigorous intensity or >6 METS). Demographical information about participants is shown in Table 4.

5. 2. 2 Procedures: A survey was created using an online, web-based survey creator (SurveyMonkey Inc., USA) and distributed to the athletic and general population cohorts via email and social media for 3 mo; of the individuals who engaged in the survey by answering at

least one question, 84% of surveys were completed in their entirety and accepted in the present study. The survey comprised twenty-five questions and aimed to identify why participants use, or do not use, OC. Initial physical activity background was established for both populations via participants being asked to provide details of the type of physical activity they undertake in an open-ended response, as well as the frequency, and intensity of these sessions.

For women using OC (womenOC), the survey inquired about the duration of use and form of OC currently being used. Participants were also asked to rank the reasons for OC use from a list of seven responses; including birth control, cycle control and reducing menstrual disturbances. They were instructed to only rank the reasons relevant to them. If participants indicated there were other reasons not included in the list, they were asked to clarify their reason in an open-ended response. If participants indicated they were not currently using OC (i.e., considered "normally-menstruating" but not necessarily regular; womenNM), they were asked to rank their reasons for avoiding OC. Participants were provided with nine potential deterrents including side effects, medical concerns or conditions, reduced exercise performance and the desire to become pregnant. If participants indicated there were other reasons not included in the list, they were asked to clarify their reason in an open-ended response.

Informed consent was attained from all participants prior to participating in the survey as approved by the Bond University Human Research Ethics Committee. All participants remained anonymous unless they wished to participate in follow-up research, in which case they were asked to provide their name and contact details.

5. 2. 3 Statistical Analysis: Chi-Square (χ^2) Test for relatedness was conducted to identify common benefits and barriers for OC use or non-use within the three physical activity groups (UT, REC, TR). Variables showing a Pearson's chi-square (2-sided) of $p < 0.05$ significance were re-tested with adjusted standardised residuals (z-scores). In the instance of a z-score of >1.96, a p-value was calculated using the standardised residual method post-hoc technique, as described by Beasley 1995 [21] and Garcia-Perez 2003[524, 525]. An adjusted Bonferroni was used to maintain an accurate family-wise alpha rate by dividing the alpha level ($\alpha = .05$) by the number of comparisons made. Our determined α_{adj} was $p < 0.00$. Results are presented as frequencies (percentages), χ^2 and p-values. Data analysis was conducted using statistical software package (IBM SPSS Statistics Version 21).

5. 3 Results

The prevalence rates of OC use were 31%, 39%, and 47% for the UT, REC, and TR groups, respectively. Despite an apparent mean increase in prevalence with physical activity level (Figure 5), there were no significant differences among the three groups ($p > 0.05$) (see Table 4).

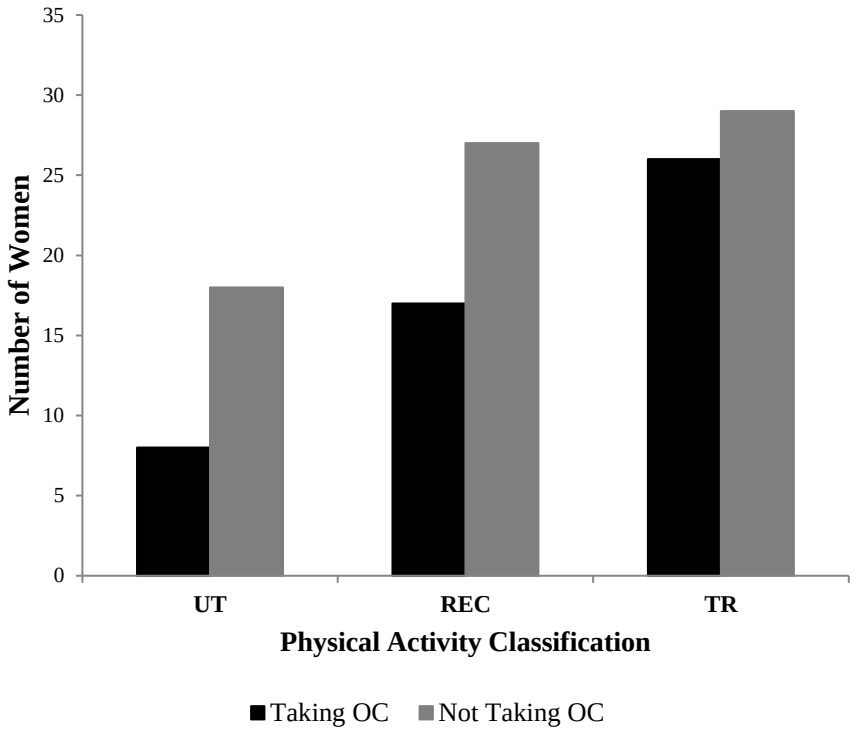

Figure 5. Comparison of women taking OC between exercise groups; UT = Untrained, REC = Recreationally Active, TR = Trained, OC = Oral Contraception.

Table 4. Subject characteristics including the prevalence of oral contraceptive use in untrained (UT group), recreationally active (REC group) and trained (TR group) women.

Subject characteristics / Group	UT (n = 26)	REC (n = 44)	TR (n = 55)
Age (yr)	32.2 ±7.3	30.6 ±6.8	27.1 ±8.3
Employment status[a]			
Full time	15	28	21
Part time / Casual	3	9	21
Student	4	7	20
Mother / Homemaker	3	3	0
Not stated	1	0	3
Currently using oral contraception			
Yes	8 (31%)	17 (39%)	26 (47%)
No	18 (69%)	27 (61%)	29 (52%)
Duration of use			
< 1 year	0	2 (12%)	1 (4%)
1 – 3 yr	1 (13%)	4 (23%)	7 (27%)
4 - 8 yr	3 (37%)	2 (12%)	5 (19%)
8 – 15 yr	3 (37%)	5 (30%)	10 (38%)
> 15 yr	1 (13%)	4 (23%)	3 (12%)
Type of oral contraceptive			
Monophasic	2 (25%)	7 (41%)	9 (35%)
Biphasic	0	3 (18%)	5 (19%)
Triphasic	4 (50%)	5 (29%)	6 (23%)
Progesterone only (i.e., mini pill)	2 (25%)	2 (12%)	6 (23%)
Type of activity [a]			
Walking	17 (65%)	15 (34%)	6 (11%)
Jogging / running	1 (2%)	18 (41%)	24 (44%)
Cycling	2 (7%)	4 (9%)	3 (5%)
Swimming / Surf-Based Sports / Rowing	4 (15%)	14 (32%)	28 (33%)
Organised sport (tennis, soccer, hockey & netball)	2 (7%)	5 (11%)	9 (16%)
Group classes / Resistance training	3 (12%)	24 (55%)	33 (60%)
Playing with children / walking pets	1 (2%)	2 (5%)	0
Housework / Gardening	1 (2%)	0	0
None	1 (2%)	0	0

. [a] More than one response allowed per person.

Universally, preventing unwanted pregnancies (90%), cycle control (86%) and reducing menstrual symptoms (88%) were the major reasons for using OC (n = 51). Additionally, a decreased risk of anaemia or bone mineral density were reported as a benefit of using OC in the UT group (50%) and a reduction in acne in the REC group (52%) and TR group (54%) (Table 5).

Table 5. Perceived benefits of using oral contraception in untrained (UT group), recreationally active (REC group) and trained (TR group) Australian women (n = 51).

Benefit	**UT**				**REC**				TR			
	Frequency	Chi-square (χ^2)	p-value	Effect Size (*w*)	Frequency	Chi-square (χ^2)	p-value	Effect Size (*w*)	Frequency	Chi-square (χ^2)	p-value	Effect Size (*w*)
Birth Control	7 (87%)	1.37	0.242	0.16	15 (82%)	0.21	0.646	0.06	24 (92%)	1.96	0.162	0.20
Cycle Control	8 (100%)	0.28	0.596	0.07	12 (70%)	1.88	0.171	0.19	24 (92%)	3.06	0.080	0.24
Reduce Menstrual Symptoms	5(62%)	1.25	0.260	0.16	10 (58%)	0.94	0.330	0.14	20 (77%)	3.42	0.060	0.26
Relief from PCOS	3(37%)	0.34	0.562	0.08	7 (41%)	0.03	0.973	0.02	9 (35%)	0.10	0.749	0.04
Reduce Acne	3 (37%)	1.72	0.190	0.18	9 (52%)	0.00	0.940	0.00	14 (54%)	1.30	0.250	0.16
Reduce Anaemia / BMD Loss	4 (50%)	0.03	0.870	0.02	6 (35%)	0.03	0.860	0.02	8 (31%)	0.00	0.970	0.00

Note: More than 1 response allowed per person. PCOS = Polycystic Ovarian Syndrome; BMD = Bone Mineral Density.

Daily commitment to taking the OC pill each day (57%), use of other forms of birth control (55%) and side effects (54%) were the major reasons for not taking OC for womenNM (n = 74) (Table 6). In the womenNM UT group, the predominant reasons for not using OC were using other forms of birth control (55%) and the commitment to take the pill each day (61%). Trained women also reported using other forms of contraception (51%) and the presence of synthetic hormones (51%) as reasons for avoiding OC. Side effects associated with OC use (66%) and a desire to fall pregnant (66%) were the predominant reasons contributing to the avoidance of OC in the womenOC REC group.

Table 6. Perceived barriers towards oral contraceptive use in untrained, recreationally active and trained Australian participants (n = 74).

Barrier	**UT**				**REC**				**TR**			
	Frequency	Chi-square (χ^2)	p-value	Effect Size (*w*)	Frequency	Chi-square (χ^2)	p-value	Effect Size (*w*)	Frequency	Chi-square (χ^2)	p-value	Effect Size (*w*)
Using other Contraception	10 (55%)	0.48	0.490	0.08	16 (59%)	0.40	0.529	0.07	15 (51%)	1.37	0.242	0.14
Side Effects	9 (50%)	0.10	0.749	0.04	18 (66%)	2.46	0.116	0.18	13 (44%)	3.17	0.075	0.21
Medical Condition	6 (33%)	0.11	0.741	0.04	15 (55%)	2.56	0.110	0.19	11 (37%)	1.61	0.204	0.15
DVT / Blood Clot	1 (16%)	0.09	0.764	0.03	3 (20%)	0.64	0.423	0.09	2 (18%)	0.25	0.617	0.06
Hypertension	2 (33%)	1.21	0.027	0.13	1 (6%)	0.49	0.482	0.08	2 (18%)	0.04	0.841	0.02
CVD	0	0	0	0.00	1 (6%)	1.96	0.161	0.16	0	0.81	0.376	0.10
Medical Concerns	7 (38%)	0.03	0.865	0.02	16 (59%)	4.12	0.042	0.24	9 (31%)	4.41	0.036	0.24
Desire to Conceive	6 (27%)	0.40	0.529	0.07	18 (66%)	5.62	0.018	0.28	11 (37%)	3.13	0.077	0.21
Commitment to take	11 (61%)	1.12	0.290	0.12	17 (62%)	0.77	0.380	0.10	14 (48%)	2.92	0.090	0.20
Synthetic Hormones	8 (44%)	0.00	0.960	0.00	15 (55%)	0.44	0.510	0.08	15 (51%)	0.45	0.500	0.08
Reduced Exercise Performance	4 (22%)	0.58	0.450	0.09	13 (44%)	3.17	0.080	0.21	9 (31%)	1.17	0.280	0.13

Note: More than 1 response allowed per person. CVD = Cardiovascular Disease; DVT = Deep Vein Thrombosis.

A reduction in menstrual symptoms was consistently reported across all womenOC (n = 51) as a benefit of taking the pill. Common menstrual disturbances reported by the women as a result of OC use are shown in Table 7.

Table 7. Commonly experienced menstrual symptoms as identified in women leading to OC use (n = 35).

Menstrual Symptom	**UT**				**REC**				**TR**			
	Frequency	Chi-square (χ^2)	p-value	Effect Size (*w*)	Frequency	Chi-square (χ^2)	p-value	Effect Size (*w*)	Frequency	Chi-square (χ^2)	p-value	Effect Size (*w*)
Heavy Period	3 (60%)	0.14	0.704	0.06	6 (60%)	1.12	0.258	0.18	3 (15%)	1.93	0.165	0.23
Painful Period	3 (60%)	0.83	0.360	0.15	7 (70%)	0.14	0.710	0.06	12 (60%)	1.21	0.270	0.19
Bloating	1 (20%)	0.77	0.379	0.15	5 (50%)	1.04	0.308	0.17	4 (20%)	0.07	0.787	0.04
Breast Tenderness	2 (40%)	0.09	0.764	0.05	2 (20%)	0.38	0.535	0.10	4 (20%)	0.12	0.726	0.06
Mood Disturbance	2 (40%)	0.05	0.830	0.04	3 (30%)	5.76	0.020	0.41	6 (30%)	0.55	0.460	0.13

Note: More than 1 response allowed per person.

The side effects and medical conditions associated with OC contributed to womenNM avoiding the use of OC (n = 40). The predominant side effects and medical conditions are shown in Table 8.

Table 8. Common side effects and medical conditions of oral contraception in women (n = 40) contributing to non-OC use.

Side Effect	**UT**				**REC**				**TR**			
	Frequency	Chi-square (χ^2)	p-value	Effect Size (*w*)	Frequency	Chi-square (χ^2)	p-value	Effect Size (*w*)	Frequency	Chi-square (χ^2)	p-value	Effect Size (*w*)
Weight gain	9 (100%)	2.4	0.121	0.24	12 (66%)	0.64	0.424	0.13	8 (61%)	4.12	0.042	0.32
Increased appetite	1 (11%)	0.36	0.549	0.09	3 (16%)	0.02	0.889	0.02	4 (30%)	0.12	0.726	0.05
Increased acne	2 (22%)	3.92	0.048	0.31	1 (5%)	0.00	0.944	0.00	0	0	0	0.00
Nausea	2 (22%)	1.17	0.280	0.17	2 (11%)	0.05	0.818	0.04	1 (7%)	1.21	0.271	0.17
Headache	3 (33%)	0.92	0.337	0.15	5 (27%)	1.77	0.184	0.21	1 (7%)	4.24	0.039	0.33
Mood Changes	6 (66%)	0.67	0.412	0.13	7 (38%)	0.14	0.711	0.06	9 (69%)	0.10	0.749	0.05
Reduced Libido	2 (22%)	0.64	0.424	0.13	9 (50%)	4.58	0.032	0.34	4 (30%)	2.07	0.150	0.23

Note: More than 1 response allowed per person.

5. 4 Discussion

The results of the present study suggest physical activity levels do not play a role in the motives for OC use in Australian women. The predominant reasons for OC use were similar between all women, with birth control, cycle control and a reduction in menstrual symptoms all considered benefits of OC use. In both REC and TR women, a reduction in acne vulgaris was a reason reported to contribute to OC use, which may reflect a greater emphasis on physical appearance in active women compared with untrained women. Untrained women considered a reduced risk of anaemia and bone mineral density (BMD) a more important benefit of OC use, likely because they are not gaining these benefits from engaging in weight bearing exercise. Similarly to the current study, a previous investigation [163] identified cycle regularity (60%), a reduction in dysmenorrhea (42%) and acne relief (37%) to be the most known non-contraceptive benefits of OC in general population women. These findings are likely attributable to the ability to manipulate the timing of menstruation or reduce adverse symptoms associated with menstruation that may impede exercise when using OC which may be beneficial to active women.

Heavy periods (menorrhagia) were reported in 60% of UT and REC women but merely 15% of TR women, suggesting an association between elevated physical activity levels and lower menstrual blood loss. Menorrhagia refers to a menstrual blood loss of >80mL per cycle [526] and is the most frequent cause of iron-deficiency anaemia in women of reproductive age [527]. Menstrual blood loss may have contributed to the higher amount of UT women reporting a decreased risk of anaemia or reduced BMD (50%) as an important benefit of OC use, particularly if they are experiencing menorrhagia frequently and are not engaged in adequate weight-bearing exercise regimes to maintain bone health. The prevalence rate of menorrhagia reportedly ranges from 5-37% within the normal population in women of reproductive age [528-530], with approximately 12% of gynaecological referrals addressing menorrhagia [531]. Conversely, secondary amenorrhea is a condition characterised by the absence of menstruation for at least three months [93, 532] and is more commonly experienced in athletes (1-50%) compared with general population women (2-5%) [97-102], further substantiating an association between physical activity levels and menstrual functioning [97-102]. The results of the present study further suggest a potential association between exercise and menstrual blood loss, with less active women more likely to take OC to attenuate menorrhagia and anaemia and subsequently potential decreases in BMD.

Painful periods were also reportedly a major side effect of the menstrual cycle and contributed to OC use in 60% of TR women. It is possible the presence of dysmenorrhea (or severe menstrual

pain) may interfere with physical activity and active women may wish to minimise this impediment. Additionally, other side effects of the menstrual cycle including bloating, breast tenderness and mood disturbances were also reported contributors to OC use in all women. Interestingly, the commitment to take OC each day was considered a common barrier in UT women (61%) and REC women (62%) compared with TR women (48%; $p > 0.05$; $w = 0.20$), which may reflect a greater amount of discipline in TR women due to strict physical activity habits, which may explain why more UT and REC women reported difficulties with committing to following the OC pill protocol.

Contrasting the perceived benefits of OC usage, the major reasons for not taking OC in TR women were the presence of synthetic hormones (51%) as well as utilising other methods of birth control (51%), with similar rates observed in UT and REC women. It is possible that women may wish to avoid ingesting unnatural substances or may have concerns about how these exogenous hormones will affect health and exercise performance. However only 31% of TR women reported a reduction in exercise performance as a barrier for OC use, which may reflect an unawareness of the association between OC and reduced peak aerobic power [7, 8, 167] and ability to recover from eccentric exercise [521, 522]. Medical conditions and concerns were considered a barrier of OC use in over half of REC women, but appear less concerning in UT and TR women, despite hypertension and deep vein thrombosis/blood clotting (DVT) reportedly experienced across all three sample groups and cardiovascular disease experienced in REC women. It is possible that the REC women in the present study reflect a wider range of women than TR or UT women, and subsequently REC women may have a higher prevalence of medical conditions or concerns in relation to taking OC as a result of larger sample representation. Recreationally active women also reported a greater desire to fall pregnant (66%; $p = 0.01$; $w = 0.28$) more than UT (27%; $p > 0.05$; $w = 0.07$) or TR (37%; $p > 0.05$; $w = 0.21$) women, which may be a further reflection of a wider range of women within the REC sample, compared with the UT and TR women. It is also possible that TR women may be preparing for upcoming competitions or events and may be less inclined to start a family until after they have achieved any potential athletic goals.

Side effects of OC were commonly reported barriers for use in REC (66%; $p > 0.05$; $w = 0.04$), UT (50%; $p > 0.05$; $w = 0.18$) and TR (44%; $p > 0.05$; $w = 0.21$) participants. As found in previous investigations [520][533], weight gain and mood changes associated with OC were considered major barriers for OC use which parallel those findings from the present study where weight gain was reported as predominant side effect in UT (100%) and REC (66%) women, while mood change was the most commonly reported side effect in TR (69%) women. These findings are in

line with a previous investigation in medical students, who found weight gain to occur in 17% of OC users and could provide reason for pill discontinuation [134]. It is possible however, that REC and TR women may be less concerned with changes in weight due to more strict dietary and exercise regimes, whereas UT women may be less inclined to adhere to a restricted dietary intake and exercise programs, and consequently this higher prevalence of weight gain. It is possible that TR women consider mood changes associated with OC to be a more important barrier for use than weight gain, as any changes to cognitive thinking and arousal may negatively affect exercise performance or the desire to be physically active on a daily basis. Taken together, these findings suggest that physical activity levels may indirectly contribute to a woman's decision to take OC.

Nausea and headaches were reported by all women as potential side effects of OC use, with increases in acne reportedly occurring in UT and REC women only, which is in line with previous research [134, 520, 533] in university students and American women reporting nausea and headaches as commonly experienced side effects of OC use. It is possible that TR women may be concerned with headaches while taking OC due to the amount of weekly exercise undertaken. Regular aerobic exercise has been reported [534] to lessen the pain, frequency and severity of headaches and migraines, and consequently TR women in the present study may experience less headaches than other women due to their physical activity levels, subsequently not considering headaches as such a barrier for OC use; further substantiating weekly physical activity levels as an indirect contributor to the decision to take OC.

The present study identified physical activity levels do not appear to play role in OC use in Australian women; however the benefits of engaging in frequent exercise may indirectly affect the perceived needs of a woman while taking OC. All women, regardless of physical activity level reported birth control, cycle control and a reduction in menstrual symptoms as major benefits of taking OC. Less active women appear to consider a reduction in anaemia a key benefit for OC use as well. The reasons for not taking OC were more diverse between the sample populations, with the predominant reasons for not taking OC in trained women being the presence of unnatural hormones or utilising other methods of birth control. Untrained women also reported using other forms of contraception and the commitment to take the pill each day as barriers for OC use. Whereas, the side effects of OC use and a desire to become pregnant commonly prevented REC women from using OC, with all sub-groups of women reporting weight gain as a potential barrier for OC use, particularly women engaged in less weekly exercise. It is possible a lack of education and awareness about the influence of OC on health and performance has contributed to the

findings of the present study, and Australian women may benefit from a greater understanding of the effect of exogenous estrogen and progesterone on physiology.

5. 5 Practical Applications

Physical activity level does not appear to play a role in OC use in Australian women; however the lack of an association is interesting given the new information regarding the effect of OC on exercise performance. The major benefits of taking OC across all women regardless of physical activity levels were birth control, cycle control and reducing adverse symptoms associated with the menstruation. In addition, UT women commonly reported taking OC reduce the risk of anaemia or reductions in bone mineral density. The major barriers towards OC use irrespective of physical activity levels were the side effects associated with the pill, the commitment to take the pill every day, and the presence of unnatural hormones, as well as the use of other methods of birth control. In the women who reported side effects associated with taking OC as a barrier, weight gain, changes in mood and a reduced libido were identified as common side effects.

CHAPTER SIX

Experimental Study Four

Salivary cortisol and IgA response to incremental cycling in a thermally-stressful environment; the role of oral contraceptives and core body temperature.

6. 1 Introduction

Cortisol is considered a very potent hormone in human physiology and can easily target most of the body's cells [181]. In high dosages, cortisol reportedly contributes to immune suppression [535]. This association between cortisol and immune function is likely attributable to the presence of neuroendocrine receptors located on several immune cells including lymphocytes [45]. Specifically, these neuroendocrine receptors can inhibit the trafficking and effector functions of B-lymphocytes [252], including s-IgA. Salivary immunoglobulin A is a form of B-lymphocyte produced in plasma cells located close to the salivary glands [424] and acts to neutralize viruses and toxins by inhibiting the attachment and replication of invading pathogens to the upper respiratory tract [427]. Salivary IgA concentration is reportedly affected by exercise with both increases and decreases observed [437, 536] depending on the intensity and frequency of the exercise undertaken. [265]. Similarly to s-IgA, it is likely that the intensity and duration of exercise also determines the magnitude of cortisol response to exercise [240] with high intensity exercise contributing to a surge in cortisol levels [237, 238, 240].

Several lymphocytes exhibit receptors for estrogen and progesterone which may affect cellular responses [38, 347] such as estrogen, enhancing the immune system [349], leading to increases in immunoglobulins [350]. In particular [s-IgA] has been shown to mimic serum estradiol throughout the menstrual cycle [34], demonstrating the influence of circulating ovarian hormones. Equivocal results have been reported regarding the effect of endogenous estrogen and progesterone on cortisol secretion at rest and throughout exercise [19, 21, 220], however exogenous secretions in the form of OC appear more apparent, leading to increases in serum cortisol comapred with women who are normally menstruating and men [21]. Considering the differences in cortisol secretion at rest between OC users and normally menstruating women, it is conceivable that the stress response to exercise and the subsequent effect on mucosal immunity would also differ and warrants investigation.

Environmental temperature is known stressor affecting SNS and HPA axis activity and may provoke a large exercise-induced cortisol response [537, 538]. Passive heating can reportedly lead

to increases in T_C of up to 1.2°C consequently causing an increase in cortisol secretion [539, 540]. It is possible that engaging in exercise in HH conditions would evoke greater increases in cortisol secretion [541], which may be amplified in women taking OC if they already experience high secretions of cortisol. If the addition of heat stress provoked a substantial rise in [sCort] throughout exercise, a suppression of [s-IgA] may occur [535], compromising the health of women exercising in warmer climates.

Endogenous estrogen and progesterone are known to affect T_C, with progesterone reported to negatively affect warm-sensitive neuron activity, consequently hindering heat-loss mechanisms [386], and likely contributes to the increase in basal body temperature of 0.3-0.5°C [73, 74, 381] observed throughout the luteal phase of the menstrual cycle. Less is known about the effect of exogenous progesterone on central regulatory mechanisms particularly in hot environmental conditions; however it is likely that exogenous hormones would cause similar changes to T_C. Throughout intense exercise a substantial amount of heat is produced as a result of the energy generated from active muscles, with this excess heat contributing to an increase in T_C [47]. The production of excess heat and subsequent increases in T_C are exacerbated if exercising in hot conditions, as the ability of the body to lose heat throughout conduction, convection and radiation is reduced [47]. It is likely that exercising in the heat would elicit a greater stress response, which may lead to substantial changes in mucosal immunity, and these changes may be exacerbated in women taking OC if they are exposed to higher cortisol secretion and T_C. It is hypothesized that exogenous progesterone will cause an increase in core temperature in oral contraceptive users, which will lead to a greater secretion of cortisol and subsequently decreases in s-IgA.

Therefore the present study aims to investigate the effect of exogenous estrogen and progesterone administration on the s-IgA and sCort response to cycling coupled with thermal stress.

6. 2 Methods

6. 2. 1 Participants: Sixteen (n = 16) recreationally active women volunteered to participate in this study, provided written informed consent to participate in this study as approved by the Institutional Human Research Ethics committee and were then categorised as either normally menstruating (n = 8, womenNM, 21.7 ±2.9yr) or taking OC (n = 8, womenOC, 22.6 ±3.5yr). Those participants in the womenOC cohort were required to have been taking combined monophasic OC for at least the prior twelve months. Those participants allocated to the womenNM group were void of any form of hormonal contraception in the twelve months prior to the commencement of the present study. All exercise trials were conducted during the early follicular phase for womenNM and throughout the active pill stage in womenOC in attempt to

achieve a stable hormonal balance between the groups and minimise menstrual disturbances such as pre-menstrual syndrome or dysmenorrhea influencing exercise performances. Normally menstruating women undertook all exercise trials within 3- 7 days of the onset of menstrual bleeding to ensure their menstrual phases was standardised. Participant's plasma concentrations of estradiol and progesterone are shown in Table 11 to confirm cycle phase.

Table 9. Descriptive characteristics (mean ±SD) for participants classified as normally menstruating (womenNM; n = 8) and taking oral contraception (womenOC; n = 8). * Significantly higher ($p < 0.05$) than womenOC.

	WomenNM	**WomenOC**
Age (yrs)	21.7 ±3.1	22.6 ±3.7
Body Mass Pre-Exercise (kg)	64.2 ±6.2	59.7 ±6.5
Body Mass Post-Exercise (kg)	64.1 ±5.7	59.4 ±6.4
% Decrease	0.2 ±1.7	0.5 ±0.0
Height (cm)	168.38 ±6.7	163.6 ±6.1
BMD (g/cm^2)	1.27 ±0.1	1.27 ±0.1
Z-Score (SD)	2.02 ±0.8	2.21 ±1.0
Z-Score (%)	113.67 ±5.7	115.11 ±6.2
BMI (kg/m^2)	22.40 ±1.7	22.00 ±1.8
Total Mass (kg)	63.07 ±5.2	59.07 ±6.1
Fat Mass (g)	16,973.33 ±2,661.3	16,208.78 ±2,777.0
Lean Mass (g)	43,081.33 ±6,806.0	39,964.22 ±4,057.5
Total body fat (%)	28.44 ±5.6	28.96 ±3.2
Resting systolic blood pressure (mmHg)	105.8 ±9.6	108.8 ±8.0
Resting diastolic blood pressure (mmHg)	67.5 ±8.8	65.8 ±6.7
Max oxygen uptake ($ml/kg/min^{-1}$)	44.3 ±5.1	44.59 ±4.0
Lactate Threshold One Power Output (watts)	80 ±25	72 ±16
Estradiol E2 (pg/ml)	91.66 ±69.29*	9.39 ±7.11
Progesterone P4 (ng/ml)	0.31 ±0.15*	0.10 ±0.05

6. 2. 2 Procedure: Each participant was required to undertake four laboratory testing sessions which included exercise pre-screening and DEXA scanning (Table 9), a lactate threshold testing to identify respective workloads and two threshold trials completed under different environmental conditions: TN (22°C; r.h. 50%) and HH (35°C, r.h. 50%); the threshold trial order was randomised for each participant. The initial testing session was to determine individual participant's LT1 via a long-graded cycling test to exhaustion [542] utilising open circuit spirometry (COSMED, Chicago, Illinois, USA) and blood lactate monitoring (Lactate Pro, ARKRAY Inc., Japan; BLa). Each subsequent trial involved cycling (Lode, Sport Excalibur,

Netherlands) at 90% of LT1 for 30 min (stage 1), 135% of LT1 for 15 min (stage 2) and then 190% of LT1 for 7.5 min (stage 3) under either the TN or HH environmental conditions.

Within one week following exercise pre-screening and the DEXA scan, each participant completed a long-graded exercise test to exhaustion to identify lactate threshold, to ensure the relative secretions of estrogen and progesterone were similar between DEXA scanning and lactate threshold testing. The lactate threshold test was conducted on a cycle ergometer (Lode, Sport Excalibur, Netherlands) with an attached programmable control unit (Lode, Sport Excalibur, Netherlands). Participants began cycling at 45 watts, with 15 watt increases every 4 min until BLa was greater than 4.0 mmol/L, as measured by a handheld automated device (Lactate Pro, ARKRAY Inc., Japan) from arterialised blood (earlobe). Upon reaching a BLa concentration of ≥ 4.0mmol/L power output was increased by 15 watts every 30 sec until volitional exhaustion. Gas exchange was monitored via open circuit spirometry (COSMED, Chicago, Illinois, USA) to identify $\dot{V}O_2max$ (L/min) (Table 9). Lactate threshold one (LT1) was identified as the power output (watts) immediately prior to an increase in BLa of 0.4 mmol/L or greater; this point was used to determine relative workloads for the cycling threshold test.

Approximately one month following the long-graded exercise test when participants were in the same phase of their menstrual or oral contraceptive cycle as the DEXA scan and lactate threshold test, they were contacted and requested to arrive at the exercise laboratory fasted (food and water) from 8:30pm the previous night. Upon arrival (approximately 0500 hours), participants were seated in a quiet, TN (21-23°C) exercise laboratory where they provided one saliva sample for baseline [s-IgA] and [sCort] (IPRO Interactive, Wallingford, UK), and one venous blood sample for estrogen and progesterone (Table 9). Whole saliva was collected via an oral swab connected to an indicator line which turned blue once 0.5ml of saliva had been absorbed. Immediately following collection, the swab was placed into 3ml of buffer solution, where it remained stable until testing. To test each sample, two drops of the buffer/saliva solution (~100 μL) are placed onto the sample pad of the lateral flow device (LFD) and set aside for ten min. Once the liquid flows across the nitrocellulose membrane, where immunoglobulin A and cortisol bind to the gold-labelled anti-IgA or cortisol antibody. Following a ten min incubation period, the LFD is placed into the LFD reader, where the relative concentrations of s-IgA or cortisol are measured within 20 sec. Participants were provided with a standardised breakfast of toast (1 slice + 0.33 per 5kg over 50kg body mass) and water (5mL/kg body mass).

Following the collection of baseline saliva samples (approximately 0500 hours), Participants undertook a 60 min acclimation period and consumed standardised breakfast of 36.9g CHO per 5kg over 50kg body mass and 5mL per/kg^{-1} body mass of room-temperature water. A second saliva sample for [s-IgA] and [sCort] was collected for pre-exercise values (approximately 0530 hours), were collected following the consumption of breakfast at approximately 30 min of acclimation. Following 60 min of seated acclimation, participants were weighed (following bladder voiding) and resting measurements T_C (HQ Inc., Palmetto, Florida, USA) were recorded while the participant was seated on the cycle ergometer. Participants commenced cycling as per previously described protocols [543]. Core body temperature was recorded at 20 sec intervals via a handheld monitoring device (CorTemp Data Recorder; HQ Inc., Palmetto, Florida, USA) for the duration of both threshold tests, with values averaged for the minute surrounding each of the time points in the threshold test. Immediately following exercise, a final saliva sample for cortisol and s-IgA was collected (approximately 0800 hours). Within one week, and while in the same menstrual or OC phase, participants returned to the exercise laboratory to undertake a second threshold trial under the opposing environmental condition. The second threshold trial was conducted identical to the first.

6. 2.3 Statistical Analyses: Distribution was assessed using the Shapiro-Wilk test for normality and boxplots with a fully-factorial ANOVA with repeated measures used to determine interactions and main effects of the three independent variables: i) Group (womenNM and womenOC); ii) Condition (TN and HH trial); and iii) Time (Rest, Stage 1-3). Where F-values were significant, least squares difference (LSD) pairwise comparisons were used. Tests of normality for salivary variables revealed the data was violated therefore a Mann-Whitney U and Friedman non-parametric equivalent test was used to determine the difference between groups (womenNM, womenOC) and time (baseline, pre-exercise and post-exercise) for [s-IgA] and [sCort], secretion rate and saliva flow rate. Data are expressed as means ±SD with alpha for statistical significance set at $p \leq 0.05$. Statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS version 22, Chicago, IL, USA).

6. 3 Results

6. 3. 1 Salivary Cortisol and IgA

No significant ($p > 0.05$) difference was identified for [sCort] (Figure 6) or [s-IgA] (Figure 7) or secretion rate (Table 12) between womenNM and womenOC at any time point under TN or HH conditions.

Table 10. Salivary Immunoglobulin A concentration ([s-IgA]) ±SD and effect size in response to exercising in 22°C and 50% r.h. (OC22 and NM22) and 35°C and 50% r.h. (OC35 and NM35).

	NM22	OC22	Effect size (Cohen's d)	NM35	OC35	Effect size (Cohen's d)
Pre-Acclimation [s-IgA] (µg/ml)	507.87 ± 427.3	644.34 ± 330.8	0.35	687.2 ± 598.1	738.77 ± 478.7	0.09
30 min Acclimation [s-IgA] (µg/ml)	257.8 ± 203.4	158.6 ± 62.5	0.65	215.71 ± 187.7	166.27 ± 87.5	0.33
Post Exercise Test [s-IgA] (µg/ml)	390.56 ± 371.0	386.38 ±103.6	0.01	510.5 ±373.8	399.5 ± 167.6	0.38

Table 11. Salivary cortisol concentration ([sCort]) ±SD and effect size in response to exercising in 22°C and 50% r.h. (OC22 and NM22) and 35°C and 50% r.h. (OC35 and NM35).

	NM22	OC22	Effect size (Cohen's d)	NM35	OC35	Effect size (Cohen's d)
Pre-Acclimation [sCort] (nmol/L)	13.62 ± 7.8	16.66 ± 9.1	-0.35	17.26 ± 5.8	20.59 ± 8.7	-0.45
30 min Acclimation [sCort] (nmol/L)	11.89 ±12.1	17.49 ± 14.6	-0.41	17.33 ± 9.7	17.88 ± 3.7	-0.07
Post Exercise Test [sCort] (nmol/L)	6.57 ± 4.1	15.4 ±10.2	-1.13	12.76 ± 11.2	15.05 ± 5.7	-0.25

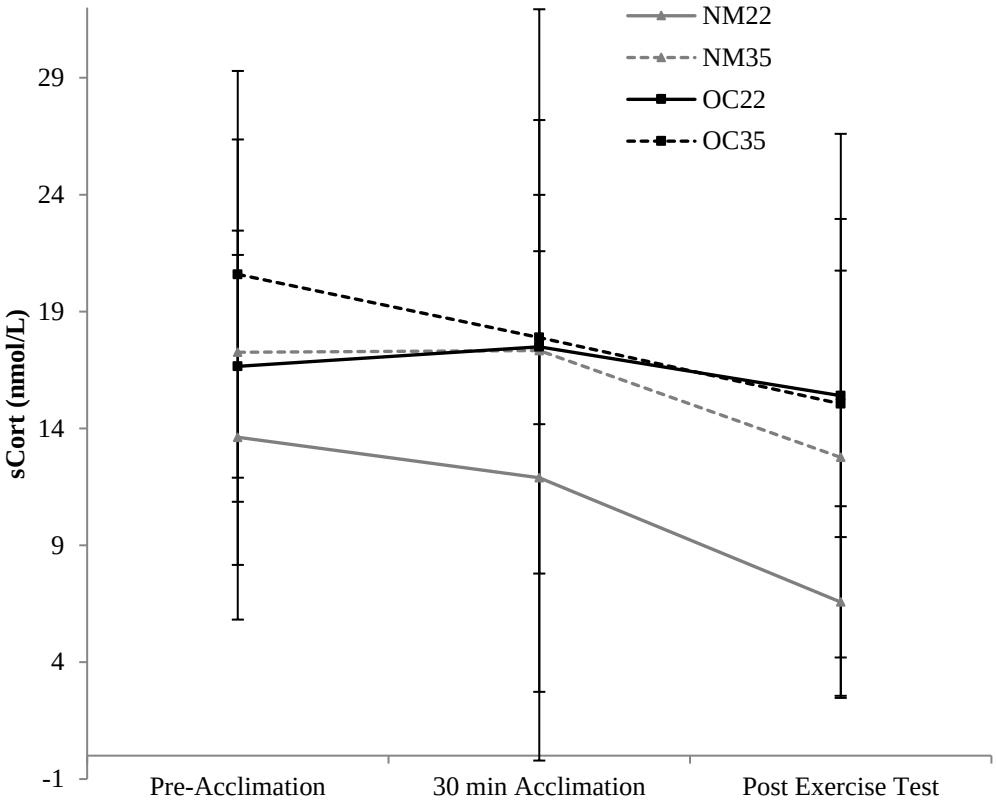

Figure 6. Salivary Cortisol concentration ([sCort]) (mean ±SD) in womenOC (n = 8) and womenNM (n = 8) participants in response to exercising in 22°C and 50% r.h. (OC22 and NM22) and 35°C and 50% r.h. (OC35 and NM35).

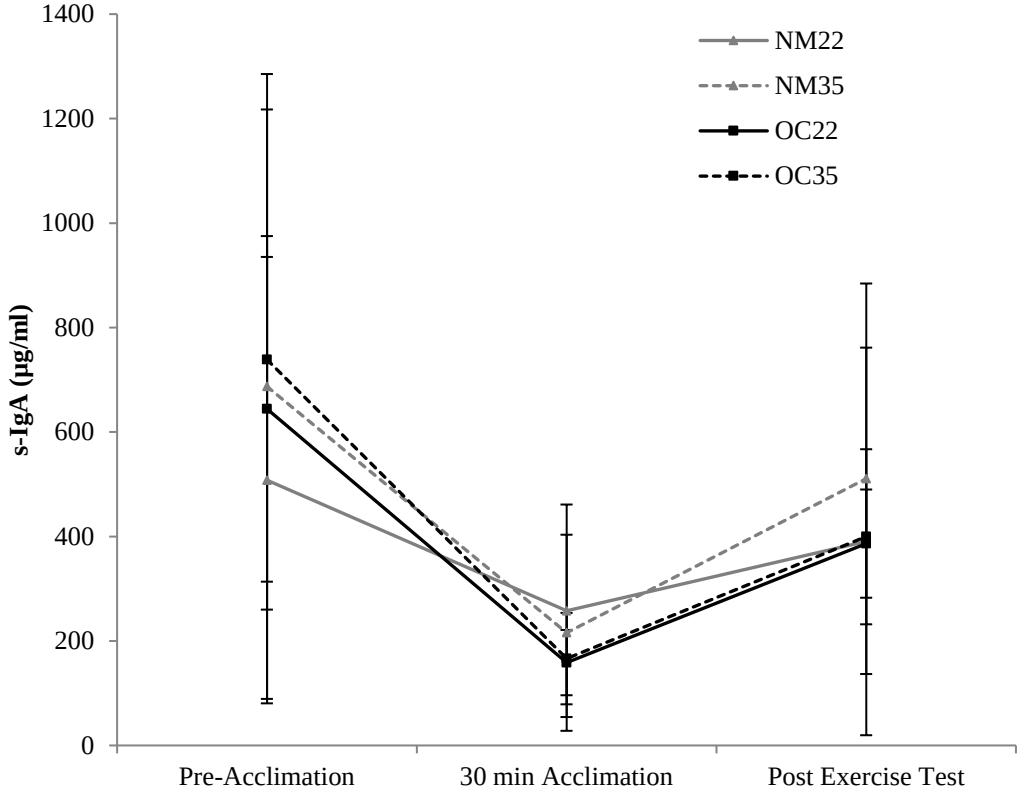

Figure 7. Salivary Immunoglobulin A concentration ([s-IgA]) (mean ±SD) in womenOC (n = 8) and womenNM (n = 8) participants in response to exercising in 22°C and 50% r.h. (OC22 and NM22) and 35°C and 50% r.h. (OC35 and NM35).

Table 12. Mean (±SD) secretion and flow rates for salivary IgA (s-IgA) and salivary cortisol (sCort) for female participants classified as normally menstruating (NM; n = 8) and taking oral contraception (OC; n = 8).

	NM35			OC35		
	Baseline	**Pre-Exercise**	**Post-Exercise**	**Baseline**	**Pre-Exercise**	**Post-Exercise**
s-IgA Secretion Rate (µg/min)	44.13 ±30.5	24.57 ±17.5	47.44 ±37.3	136.03 ±163.75	28.58 ±19.6	84.61 ±127.9
sCort Secretion Rate (nmol/min)	1.27 ±0.7	2.33 ±2.3	2.84 ±5.41	3.26 ±3.0	3.12 ±1.85	2.58 ±2.8
Flow Rate (ml/min)	0.08 ±0.1	0.14 ±0.1	0.14 ±0.1	0.16 ±0.1	0.17 ±0.1	0.16 ±0.1

Table 10. Mean salivary IgA (s-IgA) and salivary cortisol (sCort) secretion rates for female participants who were classified as normally menstruating (NM; n = 8) and taking oral contraception (OC; n = 8).

	NM22		OC22	
	s-IgA (µg/min)	**sCort (nmol/min)**	**s-IgA (µg/min)**	**sCort (nmol/min)**
Baseline	52.26 ±49.25	1.61 ±1.45	79.13 ±78.52	2.21 ±2.37
Pre-Exercise	50.31 ±43.52	1.55 ±1.68	39.99 ±32.21	3.16 ±2.93
Post Exercise	32.03 ±24.25	0.69 ±0.59	85.48 ±69.58	2.02 ±1.76

Results are shown as mean (±SD).

6. 3. 2 Core Body Temperature

Resting T_C was higher in womenOC compared to womenNM for both TN (37.35 ±0.10 vs. 37.04 ±0.21 °C; $p < 0.01$; *d = 1.88*) and HH (37.46 ±0.23 vs. 37.02 ±0.25°C; $p < 0.01$; *d = 1.83*; Figure 8) trial conditions. T_C remained significantly higher in womenOC compared to womenNM for 22.5 min in HH (37.92 ±0.22 vs. 37.65 ±0.31; $p = 0.05$; *d = 1.00*) and TN conditions (37.87 ±0.19 vs. 37.63 ±0.25; $p = 0.04$; *d = 1.08*) (Figure 8). T_C increased at every time point for womenNM in both environmental conditions ($p < 0.05$; Figure 8) and was significantly higher at 45 min (38.14 ±0.47 vs. 37.92 ±0.26°C; $p = 0.04$; *d = 0.57*) and 52.5 min (38.41 ±0.35 vs. 38.03 ±0.42°C; $p = 0.02$; *d =0.98*) in the HH trial compared to the TN trial (Figure 16). There was no difference in T_C between the TN and HH trials ($p > 0.05$) for womenOC.

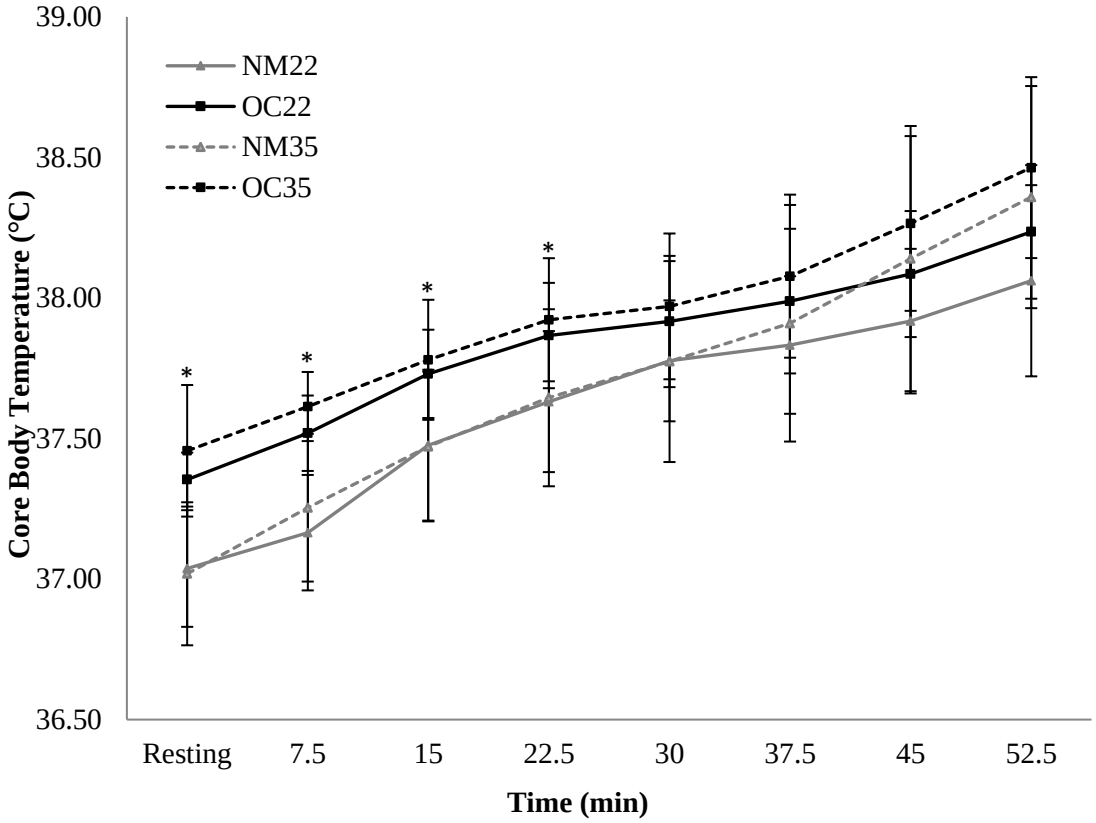

Figure 8. Core body temperature (mean ±SD) in womenOC (n = 8) and womenNM (n = 8) participants in response to exercising in 22°C and 50% r.h. (OC22 and NM22) and 35°C and 50% r.h. (OC35 and NM35). * Significant ($p < 0.05$) difference between womenNM and womenOC in both 22°C and 35°C.

6. 4 Discussion

The present study found no significant differences in [s-IgA] and [sCort] or secretion rates between womenOC and womenNM, in response to exercise in a TN or HH environment. Our findings are in agreement with previous research [544] finding exercising at $\dot{V}O_2max$ for 30 min in HH conditions (34°C: r.h. 53%) was insufficient to cause a significant change in [s-IgA] although contradicted others reporting increases in cortisol in response to repeated bouts of exercise [545] or prolonged cycling [546] and decreases in s-IgA secretion rate following 2 hr of cycling at 62% $\dot{V}O_2max$ [547], suggesting the intensity and duration of exercise have a greater influence on the [s-IgA] and [sCort] response to acute cycling, regardless of environmental temperature or oral contraceptive status. It is possible that the duration of exercise in the present study was insufficient to evoke a substantial increase in [sCort], despite the HH conditions. Considering saliva flow rate remained relatively unchanged from pre-exercise to post-exercise in

the present study (Table 12), it is possible that the exercise protocol did not cause substantial activation of the SNS; subsequently causing vasoconstriction of the blood vessels supplying the saliva glands. Despite the additional stress from the high ambient temperature. Taken together, our findings suggest the duration and intensity of exercise in the present study was insufficient to evoke a substantial changes in [s-IgA] and [sCort], as well as respective secretion and saliva flow rates.

Considering the association between elevated cortisol concentrations, decreases in the transepithelial transport of s-IgA and inhibition of B-lymphocyte synthesis [24, 254, 255, 335, 336], it was hypothesised that a decrease in [s-IgA] would occur in the present study due to high-cortisol secretions induced from exercise in the HH conditions, as demonstrated previously [547]. Conversely, our findings reject our hypothesis, demonstrating exercise in HH conditions did not evoke a substantial increase in [sCort] leading to a decrease in [s-IgA]. However, considering our final measure of [s-IgA] occurred immediately following exercise, it is possible that the true effects of cortisol on [s-IgA] were missed. Cortisol has been reported to effect B-lymphocyte synthesis for up to several days following exercise [255, 320, 346] and it is possible that any effect of cortisol on [s-IgA] in the present study had not occurred at final sample collection. However, considering no major changes in [sCort] were observed in the present study, it remains unlikely that changes in [s-IgA] would have occurred in the days following exercise.

The results of experimental study four suggest that exogenous and endogenous estrogen have similar effects on the HPA axis and subsequent cortisol response to exercise, as neither group of women demonstrated a significantly different cortisol response to the cycling exercise protocol, regardless of the environmental temperature. Even though exogenous and endogenous secretions of estrogen do not appear to affect the HPA axis differently, it remains possible that estrogen was a mechanism contributing to the absence of an exercise-induced stress response in experimental study four. It is conceivable that estrogen secretion caused a dampened HPA axis response to the exercise stimulus, ultimately not evoking a high cortisol response. Estrogen reportedly increases the production of CBG, which may explain why serum cortisol levels have been reported to increase throughout the follicular phase of the menstrual cycle or in response to taking monophasic OC, when estrogen secretion is high and consistent [21, 220]. Estrogen-induced increases in CBG lead to reduced metabolic clearance of serum cortisol while also acting as a buffer, delaying activation of the HPA axis [222]. Therefore, it is possible that estrogen contributed to an inhibition of the neural messaging to the hypothalamus, which prevented the SNS from recognising and responding to the exercise stressor. If the SNS did not receive signals

or these signals were delayed from the hypothalamus, it may be possible that the HPA axis was not sufficiently activated to evoke a substantial stress response, which may explain why [sCort] experienced a non-significant ($p > 0.05$) decrease from pre-to post-exercise in experimental study four. However, as no male subjects were investigated in experimental study four to act as controls, the influence of estradiol of HPA axis reactivity and the subsequent exercise-induced stress response remains anecdotal.

A major finding of the present study was womenOC exhibited higher resting T_C than womenNM under both environmental conditions, akin to previous investigations showing higher T_C in women taking OC compared with normally menstruating women [548] or during periods of pill withdrawal [2, 549]. In addition womenOC also maintained higher T_C than womenNM under both environmental conditions in response to exercise until 22.5 min. The mechanism behind the elevation in T_C in womenOC is likely progesterone, which reportedly causes elevations in basal body temperature of by 0.3°C - 0.5°C [74, 76, 380, 382, 550] by inhibiting the heat-loss mechanism, leading to increases in T_C by reducing warm sensitive neuron activity [47, 386, 388], which may have implications for thermoregulation during exercise.

The present study is in agreement with previous studies which have reported a blunted salivary cortisol response to exercise in response to estrogen and progesterone, which is likely to have contributed to the apparent (non-significant) up-regulation of salivary IgA following acute exercise in the present study. In addition, the present study demonstrated womenOC exhibit higher T_C at rest and in response to exercise in TN environments compared with women who are normally menstruating.

6. 5 Practical Applications

The results of the present study demonstrate acute exercise (< 60 min duration) in a HH environment does not appear to evoke a substantial change in [s-IgA] and [sCort] or secretion rates in recreationally active women. In addition, the presence of exogenous estrogen and progesterone does not appear to affect the s-IgA or sCort absolute concentrations in response to exercise in HH conditions. However, women taking OC appear to have an elevated T_C at rest compared with women who are normally menstruating and it is possible that women taking OC may need to redirect more blood flow to the skin's surface to dissipate accumulated heat, potentially compromising $\dot{Q}$.

CHAPTER SEVEN

Overall Discussion and Future Research

Experimental Study One
The Validity and Reliability for a Salivary Cortisol Point of Care Test

It is important to continually bridge the gap between controlled laboratory testing and open-field testing to allow applied sport science to evolve. Salivary analyses are gaining significant interest from the modern day sport scientist due to low costs, ease of implementation, usages and relative non-invasiveness for application within a functioning sporting organisation. Saliva is a hypotonic fluid, made up of water, electrolytes and proteins [420], with an abundance of enzymes, immunoglobulin's, hormones and other proteins in human saliva, many of which have been explored in depth using proteomic methods and identified as markers for detection of certain diseases, including oral cancers, as well as autoimmune and infectious disease [436, 551-554]. Saliva analysis has a number of applications for monitoring health and disease states as it is known to reflect serum levels of therapeutic, hormonal, immunologic, and toxicological molecules [436], as the serum components of saliva are derived from the vasculature which originates from the ceratoid arteries [446] and contain the same molecules found in the systemic circulation[420]. Oral fluid analysis may be more advantageous over serum samples due to better performance and efficiency than plasma analysis [424]. In addition, very low levels of a specific biomarker can be detected in saliva samples [436], with a total sample volume of between 0.5ml-2.0ml sufficient for accurate saliva analysis [441].

The gold standard laboratory tests for saliva analysis are the ELISA and EIA methods, both of which are a highly accurate, valid and reliable methods of determining several analytes [555] which may affect health and performance, but can be expensive and require controlled laboratory conditions. A challenge for sport scientists is to find substitute field tests, which measure the same physiological or performance parameters as laboratory testing, without compromising the integrity of the results. The two most important factors which must be considered when using a field test as a laboratory substitute, is the validity and reliability of the new testing method. For a new test or method to be considered valid, it should accurately correspond with an equivalent criterion or 'gold standard' method [556]. Demonstrating validity in new testing measures allows the sport scientist to be sure the new method is actually testing the specific physiology or performance parameter for which it is intended. In addition, the new test must also provide consistent and precise results, to allow for reproducibility [556]; this is known as reliability. Test

reliability is crucial for the sport scientist as all results should be reproducible under the same testing conditions, which allows the interpretation of any changes in results to be attributed to variables that have been manipulated i.e. training status or intensity of exercise. Experimental study one found the IPRO LFD method to be a valid point of care test for salivary cortisol when compared to Salimetrics ELISA analysis. Additionally, the OFC and LFD showed good inter-tester and device reliability.

A number of factors can affect saliva composition, including the source of saliva (whole saliva or individual glands), flow rate, nature of the stimulus, diurnal and hormonal variations and the degree of hydration of the subject [430, 466, 467]. There are several methods of collecting saliva in applied sport science research, namely cotton swabs [293, 557], passive drool [500, 558] and suction tubes [559], however it is important to consider the effect of various saliva collection methods on obtained results. The present study demonstrated the IPRO oral fluid collector method showed less variability with the Salimetrics cotton swab method ($p = 0.881$; $r = 0.33$) compared with the Salimetrics passive drool technique ($p = 0.145$; $r = 0.32$) suggesting the location of sample collection may play a role in the concentration of analytes obtained throughout testing. The three major salivary glands which produce saliva into the oral cavity are parotid, submandibular and sublingual [420] with each gland secreting different mixtures of serous-and mucinous-derived fluid, and therefore the levels of proteins can change depending on the location of saliva collection in the oral cavity [420], which may have contributed to the findings in experimental study one. The use of cotton swabs for sample collection may encourage consistency in the saliva collection process as swab placement can be standardised i.e. on top of the tongue. It is possible that the relative contribution of saliva (and subsequently, secretion of analytes) from each saliva gland could vary when passive drool techniques are utilised, which may explain why IPRO OFC was more closely correlated with SOS compared with PD in the present thesis.

For most studies investigating the human mucosal immune system, saliva has been the mucosal secretion of choice, due to the non-invasive ease of collection [451]. In addition saliva analysis does not require experienced personnel to test and can be readily used for diagnostic and health surveillance purposes [436] in applied sport science research, which is why saliva has been utilised throughout the present thesis. Experimental study one supports an innovative point of care test for the accurate analysis of salivary cortisol in applied sport science research and monitoring, which substantially decreases sample analysis time and preparation and will help to ‘bridge the gap’ between laboratory and field testing, by allowing salivary cortisol to be easily monitored anywhere, with the accuracy of gold-standard laboratory testing.

Experimental Study Two
The Response of Surf Life Saving Competition on Salivary Immunoglobulin A

The interaction between exercise and the immune system remains equivocal in applied sport science research, with conflicting reports of increases and decreases in the concentration of salivary immunoglobulin A following intense or prolonged exercise. Most authors agree that the intensity of exercise will determine the subsequent s-IgA response, with moderate intensity exercise eliciting a positive response and increasing [s-IgA], while intense or strenuous exercise reportedly decreases [s-IgA] in athletes. Upper respiratory tract infections have been identified as the most predominant infection experienced in highly trained athletes [560, 561], likely attributable to the substantial amount of time being spent in close quarters with teammates in the change rooms, at training and throughout travel [562]. This high prevalence of URTI in athletes may be exacerbated in water-based athletes, as they are consistently exposed to both airborne and waterborne pathogens which may cause infection. The relationship between athletic training and the subsequent change in [s-IgA] has been explored in several water-based sporting disciplines such as swimming and kayaking [306, 307, 315, 563, 564], however less is known about the [s-IgA] response specifically to athletic competition. Experimental study two is the first investigation into the s-IgA response surrounding a single multidisciplinary endurance SLS event. The results of this study demonstrated females and males respond similarly in the 24 hr following competition, however males experienced a significant ($p < 0.05$; $d = 1.66$) increase in [s-IgA] 61 hr post-event, suggesting that the immediate recovery protocols following SLS elite competition can be similar for males and females without increasing the risk of URTI. However 'return to training' protocols may need to be managed specifically in relation to genders. Although the findings did not reach statistical significance, the [s-IgA] in male athletes plateaued from 24 – 48 hr post-event and prior to the significant increase observed at 61 hr post-event. Whereas the female SLS athletes maintained a similar pattern of [s-IgA] from two days pre- to 61 hr post-event, with no significant ($p > 0.05$; $d = 1.66$) differences between any time points. These findings suggest that female SLS athletes experience less variation in s-IgA than male SLS athletes. However considering all saliva samples were collected throughout a taper and recovery period, it is possible that these results do not provide a true reflection of the genuine differences in [s-IgA] between male and female SLS athletes. Rather [s-IgA] may have remained relatively stable until normal training sessions were resumed at 61 hr post-event, when we observed the spike in males only.

Considering SLS athletes train for several years to achieve the physical, technical and psychological characteristics which are required to successfully compete at the elite level, it is possible the athletes may have been experiencing changes to normal mucosal immune function in the weeks leading up to testing, which may explain the discrepancies between the findings of experimental study two and previous research [306, 307, 313]. Several studies of training periods have reported decreases in [s-IgA], suggesting there may be a cumulative effect of exercise on mucosal immunity. It is possible that high intensity training over a prolonged period could cause a suppression of s-IgA, due to inadequate recovery between exercise sessions or insufficient dietary replenishment. In addition, a positive association between the intensity of the exercise and the degree of immune suppression has been identified [565], potentially attributable to the HPA axis which is stimulated in response to various stressors, including intense exercise. The SLS event investigated in experimental study two lasted up to 90 min in duration, with final placing's crucial in determining 'point' allocation for subsequent races and the overall SLS champion for that particular season. Athletes navigated their way through unpredictable surf conditions in an attempt to be crowned the winner of the event, likely evoking a substantial response from the HPA axis. When the HPA axis is activated in response to intense exercise, the SNS is also stimulated [463] which can lead to an increased transportation of s-IgA into the oral cavity [506] particularly when the metabolic demands of exercise require an increased $\dot{Q}$ [566]. Interestingly, SNS activation is reportedly less pronounced in trained individuals [567, 568]. Considering increased SNS activity is associated with an increased metabolism of the pIgR [569, 570], leading to an increased transportation of s-IgA into the oral cavity [463]. It is possible that trained individuals do not experience the same exercise-induced sympathetic nervous system activation as untrained individuals. Subsequently the mobilisation of the pIgR is not enhanced in the same manner as less trained individuals. Which may in-part explain why elite athletes have been shown to exhibit lower [s-IgA] than recreationally active individuals and perhaps why the increase in [s-IgA] observed immediately post-event compared with pre-event in the present study did not reach statistical significance. The autonomic nervous system is predominantly responsible for the secretion and composition of saliva [256, 470]. Parasympathetic stimulation of saliva generally results in a high flow rate with low levels of organic and inorganic compounds [424], while sympathetic stimulation of saliva generally has a lower flow rate, with higher levels of proteins [454]. It is important to note that a circadian rhythm for salivary flow rate has been previously identified, with the highest flow being at 0400 hours and the lowest at 2000 hours [571, 572]. Considering the timing of sample collection leading up to the event occurred at early morning training sessions (approximately 0500 hours) and the event-day samples occurred later in the day, it is possible circadian rhythms may have contributed to the secretion of s-IgA. In addition, it is

possible that the non-significant ($p > 0.05$; $d = 1.66$) increase in [s-IgA] in both males and females following the event is attributable to saliva stimulation from salt water as the competition was conducted in the ocean.

An important mechanism of maintaining normal immune health is adequate recovery following stressful exertion such as SLS competition. Considering the well-established gender differences in human physiology, it is plausible that the immune system also responds and recovers differently to exercise in male and females. Although not significant ($p > 0.05$; $d = 1.66$), [s-IgA] from 24 – 61 hr post-competition demonstrated different patterns between male and female SLS athletes, with the lack of significance potentially a reflection of sample size. A potential mechanism explaining why there does not appear to be a substantial difference in the pattern of [s-IgA] in the 24-61 hr period following SLS competition may also be attributable to the menstrual status of the female SLS athletes. Considering the physiological and psychological demands of open-ocean training and competition, it is possible that female SLS athletes may have experienced menstrual dysfunction from chronically elevated CRH leading to a suppression of GnRH, ultimately preventing endogenous estrogen secretion; a condition known as secondary amenorrhea. If several of the female SLS athletes were experiencing secondary amenorrhea at the time of testing, it is possible that any estrogen-induced enhancement of [s-IgA] would not have been evident, which may in-part explain why the male and female SLS athletes in experimental study two did not exhibit significantly different [s-IgA] from 24-61 hr post competition (Figure 4). Similarly, no differences in [s-IgA] between males and females were reported in response to cycling, swimming, marathon and ultramarathon events [284, 305, 573-576], suggesting highly trained female athletes may not exhibit the same fundamental gender-differences in [s-IgA] as less trained women, who have been shown to secrete less s-IgA than males at rest [284, 577]. Even though long-term, intense training may reduce the endogenous estrogen-enhancing effect on [s-IgA], it is possible that women may elect to use OC to simulate a normal menstrual cycle, ultimately increasing their estrogen exposure and potentially regaining enhance mucosal immune function, however information on menstrual status and OC history was not collected at the time of testing, so this remains ancedotal until further research is conducted into the effects of OC of the mucosal immune system.

Experimental Study Three
Prevalence and the associated Benefits and Barriers for Oral Contraceptive use in Australian women grouped or physical activity level.

Oral contraceptives are widely used in the general population [578, 579] with reports of more than 60 million women worldwide taking some form of OC [162]. Additionally, OC use is becoming more common in the athletic population [112, 519], potentially to re-create or control the menstrual cycle. Considering the rising prevalence of active women taking OC [112, 519], and the potential effect exogenous estrogen and progesterone may have on the mucosal immune system and exercise tolerance, it is interesting to explore whether the decision to take OC is related to physical activity levels, or if there are other factors which determine motives for OC use. Experimental study three suggested OC prevalence progessively increases with weekly physical activity levels, however this finding did not reach statistical significance ($p > 0.05$) but does suggest that Australian women may be unaware of the influence taking OC may have on exercise tolerance and performance. The most common reasons for taking OC were similar for all women, regardless of physical activity levels with birth control and cycle control the predominate reasons for taking OC. Which is in line with previous research reporting [160, 580, 581] cycle control as the most commonly identified non-contraceptive benefit of taking OC. Interestingly, the perceived barriers for OC use appeared to differ between women, with UT women reporting the commitment to take the pill and using other forms of contraception as the predominant reasons they do not use OC. Recreationally active women commonly reported side effects of the pill and a desire to conceive as barriers, while TR women reported the presence of synthetic hormones and the use of other contraceptive methods prevented them from taking OC. Even though, the differences in barriers for OC use cannot be unequivocally attributed to physical activity levels, it remains possible that exercise and lifestyle has contributed, at least in-part, to the findings from experimental study three. Untrained women reported the commitment to take OC every day as the main barrier of OC use, and while this was also acknowledged as a barrier for use in REC and TR women, it was not the most prevalent response for those women, which may reflect a greater discipline in REC and TR women from adhering to consistent physical activity and potentially dietary regimes. Trained women appeared more concerned with ingesting synthetic hormones, which may also reflect a healthy lifestyle, which TR women are likely to exhibit.

Even though side effects were one the most prevalent reasons for not taking OC in REC women, they were also commonly reported by TR and UT women as barriers for OC use. The results of experimental study three identified weight gain as the most common potential side effect of OC use in UT and REC women, and to a lesser extent, in TR women as well; however it is likely that the lifestyle of TR women may attenuate some of the weight gain associated with OC use, which

may be why weight gain appears to be less concerning to TR women. It is possible that UT and REC women, do not adhere to the same strict dietary regimes as TR women and due to their physical activity levels, weight gain may not solely be attributable to taking OC. Untrained and TR women both commonly reported mood disturbance as an OC-associated side effect which deterred them from taking the pill. It is possible that mood disturbance may affect the woman's motivation and cognitive ability throughout training, which may be irritating and interfere with exercise, posing a substantial deterrent of taking OC in TR women. Conversely, a lack of physical activity, which is known to adversely affect mood [582-584], combined with the OC-associated changes in mood, may have contributed to the finding in UT women. Other commonly identified side effects of OC in experimental study three which pose a barrier to OC use included increases in appetite, reduced libido, increased acne vulgaris and nausea, however these symptoms don't appear to be directly affected by physical activity levels and may be attributed to the OC pill or other external factors.

In addition to the potential side effects associated with taking OC, several menstrual symptoms were identified in all women which may contribute to OC use. Painful periods were the predominant menstrual symptom reported regardless of physical activity levels. Untrained and REC women also reported heavy periods as a commonly experienced menstrual symptom, which contributed to their decision to take OC, while TR women appeared more concerned with changes to mood. Trained women may be less concerned about heavy periods as a decrease in menstrual blood flow is often associated with prolonged exercise, particularly high intensity exercise. It is possible that TR women were not experiencing heavy periods to the same extent as UT or REC women due to their training status, and therefore they did not consider reducing heavy periods a major benefit which contributed to taking OC, rather changes to mood was a priority as this may affect their desire to participate in exercise.

Even though the prevalence of OC use in women is rising [162, 578, 579], women have been shown [161-163] to be poorly informed about the risks and benefits of OC, particularly in regards to health [585] and therefore may also be unaware of the effect OC can have on exercise performance, which may have in part contributed to the findings of experimental study three. Ethnic background appears to be a factor contributing towards knowledge of risks and benefits of taking OC, with Finnish women reportedly [586] less aware of the increased risk of thrombosis during OC use as well as the associated contraindication of smoking while taking OC in women above the age of 35 years. African-American women were found to be uninformed about the side effects associated with taking OC, with only 42% of women able to identify a single medical risk

associated with OC use [587]. In addition to ethnicity, age and education have also been suggested [588, 589] as factors contributing to a woman's knowledge regarding the risks and benefits of OC use. The health risks of combined OC use include an increased likelihood of venous thromboembolism [111, 156] and ischaemic stroke [111, 152], as well as an elevated risk of developing breast [117, 138, 153] and cervical cancer [117, 138, 154]. In addition, COC may also cause fluctuations in body weight and fluid retention, as well as breast tenderness, nausea, weight gain, headaches, skin problems, abdominal bleeding and ovarian cysts [111, 134, 590-592]. The women sampled in experimental study three reported medical concerns, including deep vein thrombosis, cardiovascular disease, and hypertension to be barriers towards taking OC. Interestingly, medical concerns were more commonly identified barriers in REC (59%), compared with UT (38%) and TR (31%) women, which may reflect the general health status of the REC women compared with other women in experimental study three. However, if this is the case, it is thought-provoking that UT women did not express the same concerns, due to their increased risk of health problems associated with a lack of physical activity.

There are several health risks associated with taking OC, and there are also numerous advantages namely, attenuating dysmenorrhea, acne vulgaris, iron-deficiency anaemia, pelvic inflammatory disease, as well as some cancers such as endometrial, colorectal and ovarian [111, 117, 138, 593, 594]. A reduction in menstrual symptoms was the third most commonly reported reason for taking OC across all groups of women in experimental study three. Considering common menstrual symptoms such as dysmenorrhea reportedly occur in up to 80% of the general population [595], it is not surprising that women in our study identified a reduction in menstrual symptoms as a common benefit of taking OC. Additionally, a reduction in acne vulgaris was also commonly reported in REC (52%) and TR (54%) women as a benefit contributing to OC use, while only 37% of UT women reported taking OC to lessen the symptoms. It is possible that the age of participants in experimental study three contributed to this finding, as the UT women were slightly older than the REC and TR women, and may not suffer from symptoms associated with acne vulgaris to the same extent as younger women. Oral contraceptives have also been associated with decreasing uterine fibroids, the symptoms associated with premenstrual syndrome and endometriosis, as well as rheumatoid arthritis and may lead to increases in BMD [111, 117, 138]. Interestingly, UT (50%) women reported taking OC to reduce the risk of anaemia and BMD loss more than REC (35%) and TR women (31%). It is possible that REC and TR women may be less concerned with BMD as they are more likely to engage in frequent weight-bearing activities, and may be more inclined to follow stricter dietary regimes which ensure adequate iron intake to maintain bone health.

The results of experimental study three were unable to identify a significant association between physical activity levels and OC use in Australian women; however, it is interesting to consider that the amount of weekly exercise undertaken may unintentionally impact on the perceived needs of the woman. The results of experimental study three suggest TR women are less concerned with the BMD-enhancing effects of OC use than UT women, which may be attributed to higher physical activity levels in TR women. Similarly, UT and REC women reported the commitment to take the pill everyday as major barriers to OC use more often than TR women, which may reflect a greater discipline in TR women, as a result of training commitments. A further example of the way weekly exercise levels may unknowingly contribute to OC use is commonly reported side effects of OC, in particular weight gain, which was more concerning to UT women compared with TR. This may be a reflection of lifestyle rather than OC-induced side effects, particularly considering 3G OC have been shown [596, 597] to have caused far fewer changes in weight compared with earlier generation OC. Taken together these examples demonstrate how exercise may impact on the perceived needs of women and may influence what women believe to be benefits and barriers towards OC use, even though the major reasons why women take or do not take OC appear to be relatively similar regardless of physical activity levels.

Experimental Study Four
Salivary cortisol and IgA response to incremental cycling in a thermally-stressful environment; the role of oral contraceptives and core body temperature.

Considering the findings of experimental study three and previous research [159, 160, 514] which suggest active and athletic women are taking OC just as frequently as women in the general population, it is important to investigate the way exogenous estrogen and progesterone secretion affects exercise tolerance, including the immune and stress response. Exercise is known to cause changes in normal mucosal immune function as well as stress levels depending on the intensity and duration of activity undertaken. Considering no significant difference in the [s-IgA] response to acute SLS competition was observed between males and females in experimental study two, which may have been attributed to their ‘elite’ training status or prolonged exercise-induced menstrual dysfunction, it was evident that recreationally active women may provide a more transparent insight into the acute mucosal immune and stress response to exercise, and were therefore utilised in the final two studies of the present thesis. Additionally, recreationally active women represent a larger population than elite athletes, which allows our findings to have greater practicality and applicability.

Experimental study four found no significant difference in [s-IgA] or [sCort] between womenNM and womenOC in response to acute cycling exercise, suggesting the stress and mucosal immune responses following short duration (< 60 min) exercise is not affected by the presence of exogenous estrogen and progesterone regardless of the environmental conditions. It is possible that 3G OC which contain substantially less amounts of estrogen and progesterone compared with earlier generation pills [115], may have contributed to this finding. The relative concentration of exogenous estrogen and progesterone in third generation OC has decreased four-and ten-fold from second and first generation pills respectively [37], and it is therefore possible that this reduction in the concentration of exogenous hormones has reduced some of the adverse effects of OC on health.

The duration of exercise reportedly determines the subsequent cortisol response, with long duration (> 60 min) exercise shown to cause increases in serum cortisol, whereas acute exercise is associated with minor or no changes [598] in serum cortisol levels. It is possible that the duration of exercise in experimental study four (52.5 min) was insufficient to cause a substantial rise in cortisol secretion. In addition, the intensity of exercise also appears to contribute to the cortisol response, with exercise intensities of < 50% $\dot{V}O_2$ max reportedly [599, 600] insufficient to elicit a substantial increase in cortisol secretion. It is possible that the three-tier intensity protocol utilised throughout the exercise trial in experimental study four was insufficient to cause an increase in salivary cortisol secretion. Participants were cycling at 90%, 135% and 180% of their individually determined lactate threshold for 30 min, 15 min and 7.5 min respectively.
Experimental study four identified a (non-significant; $p > 0.05$) post-exercise increase in [s-IgA] regardless of OC use indicating acute exercise can up-regulate mucosal immunity in recreationally active women, suggesting short bouts (< 60 min) of exercise at varying intensities to have a beneficial effect on the mucosal immune system. Additionally, we found short duration, acute exercise was insufficient to cause an elevated stress response, suggesting regular physical activity, with adequate rest in between sessions and/or bouts may not have cortisol-induced adverse health consequences and is therefore unlikely to cause substantial changes to menstrual cycle functioning in healthy women. Importantly, the use of OC did not affect the cortisol or s-IgA response to exercise or resting levels suggesting modern-day OC use may not elicit adverse effects on mucosal immunity and stress as previously associated with earlier generation pills. This could have implications for recreationally active women who would like to take OC but choose not to, due the potential exercise-induced implications on mucosal immunity and cortisol secretion, and the subsequent effect on exercise performance which was identified as a barrier for

OC use in 44% of recreationally active women sampled in experimental study three of the present thesis. Experimental study four demonstrated no significant difference in the mucosal immune or cortisol response to acute exercise in women taking OC compared with normally menstruating women, concluding exogenous estrogen and progesterone does not adversely affect the stress and mucosal health of the recreationally active woman in response to acute exercise in TN or HH environments and should not be considered a barrier towards taking OC in recreationally active women.

Even though taking OC does not appear to affect the s-IgA and sCort response to exercise, it does appear to affect T_C and the perceived demands of acute exercise. The results of experimental study four showed womenOC exhibit higher T_C at rest and in response to incremental cycling exercise up to 22.5 min in both TN and HH conditions. Previous research has demonstrated women taking OC exhibit higher T_C than women who are normally menstruating. This elevated T_C is likely attributable to the higher secretion of exogenous progesterone in OC compared with endogenous secretions throughout the normal menstrual cycle, which has been reported to inhibit heat-loss mechanisms. Additionally, exogenous estrogen has been shown to suppress endogenous estrogen, reducing the antagonistic effect of estrogen on lowering T_C, particularly if exogenous estrogen does counteract the effect of progesterone on T_C in the same manner as endogenous estrogen.

Following 22.5 min of exercise in both conditions, no significant difference in T_C was observed between womenNM and womenOC suggesting even though womenOC experience higher T_C in the initial stages of exercise, thermoregulatory mechanisms may be initiated earlier, consequently attenuating the progesterone-induced increases in T_C and allowing womenNM and womenOC to approach similar peak T_C towards the final stages of the exercise protocol. This finding of similar patterns of T_C beyond 22.5 min of exercise in experimental study four, may also in-part explain why there was no significant difference in [sCort] and [s-IgA] post-exercise between womenNM and womenOC. It is possible that any exercise-induced stress from cycling in 35°C in experimental study four was similar between groups as they experienced comparable T_C from 22.5 min of exercise.

Limitations of Research

It is important to acknowledge the limitations to research. A noteworthy limitation in experimental study one is an apparent overestimation of salivary cortisol concentrations below 4 mmol and above 8 mmol and an underestimation of salivary cortisol concentrations between 4 and 8 mmol. This apparent under-and over-estimation of salivary cortisol values, is likely

attributable to small sample size in experimental study one. Similarly, the outlier which is evident in Figure 2 would suggest the IPRO method may underestimate salivary cortisol concentration. However, considering this trend is only evident in one participant it may be simply due to daily fluctuations, and future studies with larger sample sizes should be conducted to confirm or refute the findings of experimental study one. In addition, daily perceived stress levels may have also contributed to variations in salivary cortisol concentrations from trial one to trial two. Considering participants did not undertake a perceived stress questionnaire or any other measures of daily perceived stress levels, it is possible external factors such as mood changes, work or study, emotional stress etc. were dissimilar in participants between the trials, contributing to variations in results and potential over-and under-estimations of salivary cortisol concentrations.

The major limitation of experimental study two was the absence of information regarding menstrual cycle and oral contraceptive practices of the female surf lifesaving athletes. This information would have assisted in enhancing or challenging the argument of an interaction between ovarian hormones and salivary immunoglobulin A. In addition, a measure of perceived workload i.e. rating of perceived exertion was not included in experimental study two, which may have provided valuable insight into the perceived physical stress of training and competition.

While the participants in experimental study three completed a survey in which they were able to provide open-ended responses for clarity if necessary, there was no forum utilised in which the researchers and the participants undertook interviews or open discussions to gather more in-depth reasoning behind each response. Furthermore, a larger sample size would have assisted in a greater insight into the perceived benefits and barriers of OC use in Australian women and allowed for a more representative study.

Experimental study four collected saliva samples for [sCort] and [s-IgA] prior to-and immediately following acute exercise. The intensity of exercise within this acute bout was altered three times, however no saliva samples were collected during exercise when exercise intensity increases and is considered a limitation of the study. Another limitation to experimental study four, in that the final saliva sample for [s-IgA] and [sCort] occurred immediately following the cessation of exercise, and considering cortisol has been shown to influence the synthesis of B-lymphocytes for up to several days following intense exercise [255, 320, 346], it is possible a delayed effect of exercising in a HH environment may have occurred. However, considering [sCort] did not exhibit a substantial rise post-exercise in experimental study four as demonstrated following acute resistance exercise [243-245]; it is unlikely that a delayed effect of cortisol-induced suppression

on [s-IgA] would have occurred in the days following the exercise trial. Rather the results of experimental study four indicate that acute exercise (i.e. < 60 min in duration) with a three-tier intensity increase does not evoke substantial changes in [s-IgA] or [sCort] even with the addition of thermal stress, with the use of OC having no effect.

Finally, the time of testing in experimental study four may have influenced our findings as womenOC and womenNM were exposed to varying levels of estrogen and progesterone as womenNM were testing throughout the early follicular phase of the menstrual cycle while womenOC were tested while throughout the active pill phase of their OC cycles (Table 11). While this presents a limitation to experimental study four, it is important to consider the practicality of our study in relation to recreationally active women. Women taking OC will be exposed to exogenous estrogen and progesterone secretion (active pill phase) for at least 21 days per month, with the T_C response to exercise most applicable throughout the active pill phase as womenOC are likely to be exercising throughout this time more often than throughout menstruation or the inactive pill phase. While womenNM are more likely to be affected by pre-menstrual symptoms which may affect the desire and ability to exercise as observed in experimental study two; these symptoms may begin to present in the late-luteal phase of the menstrual cycle, as endogenous progesterone levels are beginning to decrease. It therefore appeared practical to test normally menstruating women following pre-menstrual symptoms or once other disturbances associated with menstruation had ceased or were unlikely to occur, and subsequently were tested throughout the early follicular phase of the menstrual cycle.

Future Research

Further research should be conducted to identify the interaction between exogenous estrogen and progesterone administration on exercise tolerance in active women in response to prolonged exercise. Future studies should consider testing women in all phases of the menstrual cycle, to account for variations in the secretion of endogenous estrogen and progesterone and should include males as controlled participants. Similarly, women taking OC should be tested in both the active and inactive pill phases in monophasic OC users, as well as the periods of varying hormone secretion in biphasic and triphasic users. Progesterone-only (mini pill) users should also be considered as important participants, particularly when investigating exercise in HH conditions and monitoring changes in T_C. Finally, research should consider both recreationally active women and female athletes, to explore the true effect of OC use on exercise tolerance.

The long term effects of OC administration on [s-IgA] and [sCort] should also be explored. Previous research has suggested taking OC could lead to an increase in cortisol secretion, which if occurring chronically over several years of OC use, may have serious implications for the health of the woman. Additionally, the interaction between exercise-induced increases in [sCort] during OC use warrants further investigation as additional increases in [sCort] may ultimately affect exercise performance, if participating in exercise of a prolonged duration. Similarly [s-IgA] should be monitored over long-term OC use due to the known relationship between endogenous estrogen and immune function. If this relationship does not occur with exogenous estrogen, and considering exogenous estrogen is known to completely suppress endogenous secretions, it is possible that OC use may lead to compromised mucosal immunity, which may be exacerbated in active women, if engaging in frequent, high-intensity exercise.

The thermoregulatory response to prolonged exercise in TN and HH conditions must also be explored further. The present thesis has demonstrated significant differences T_C between women taking OC and women who are normally menstruating at rest and in response to acute exercise. If OC use increases T_C as suggested in the present thesis, it is likely that the initiation of thermo-regulatory mechanisms is also different, which may have significant implications for exercise performance, as effective thermoregulation places substantially more demand on cardiac output, potentially compromising exercise performance. It is therefore imperative that future research is conducted into the thermo-regulatory mechanisms and demands of exercising in TN and HH environments in active women taking OC compared with normally menstruating women, and if

these factors are responsible for the increased perceived exertion throughout exercise in women taking OC.

CHAPTER EIGHT

General Methodology

8. 1 Individual Profiling (IPRO) Saliva Analysis

8. 1. 1 Background

Traditionally, saliva analysis for the purposes of laboratory testing has proven expensive and time consuming, however, a portable saliva analysis system has been developed which allows for point of care testing in real time. The demand for instant feedback is ever-growing, particularly in applied sport science research, where the effective relay of information could be the difference between winning and losing. Gleeson et al. [307] pioneered the integration of salivary analysis to determine athlete tolerance to the demand of training and competition and established for measures of [s-IgA] to be beneficial to athletes and coaches, the results must be rapidly accessible, as athletes may continue to train oblivious to the immunosuppression which may be occurring. The Individual Profiling point of care saliva analysis kit (IPRO Interactive, Oxfordshire, UK) represents and novel salivary analysis method for swift feedback for coaches and athletes, and could prove advantageous for training and recovery purposes [488]. Furthermore, the IPRO system is relatively inexpensive and could be a suitable method for use within sporting organisations [490]. The IPRO system has passed validation tests which concluded the device is suitable for the collection and testing of saliva using real time analysis [601]. Furthermore the IPRO interactive system has been shown to be in good agreement with the gold-standard ELISA method, for determining salivary IgA [490, 602] and cortisol (Chapter Three) levels.

8. 1. 2 Equipment

The IPRO device consists of an OFC synthetic polymer-based swab attached to a volume adequacy indicator stem (Figure 9A). The indicator stem is designed to turn bright blue upon the collection of approximately 0.5mL of saliva. Following collection, the OFC swab is placed into a dropper bottle containing 3mL of extraction buffer (Figure 9B). The extraction buffer is comprised of sodium phosphate, salts, detergents and preservatives, which act to draw the target analytes from the OFC swab into the buffer and to prevent the growth of microorganisms. Prior to sample analysis, the dropper bottle containing the saliva sample is lightly shaken for two min [601]. The sensitivity of the IPRO device is 10 µg/mL and the dynamic range of the device is 18.75 µg/mL to 1200 µg/mL.

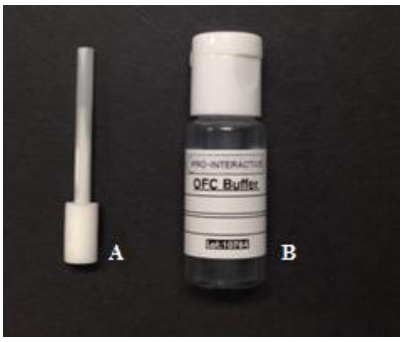

Figure 9. (A) IPRO OFC Swab; (B) IPRO dropper bottle containing buffer solution.

8. 1. 3 Procedure

The buffer bottle is held perpendicular to the LFD (Figure 10C) and two drops of the buffer/saliva solution (~100 μL) are placed onto the sample pad of the LFD and set aside for ten min. The liquid uses a capillary action to travel through the conjugate pad hydrating the dried conjugate (Figure 10B) i.e. the liquid flows without the assistance of, or in opposition to external forces such as gravity through a narrow space. The liquid continues to flow through the nitrocellulose membrane towards the wicking pad at the end of the strip. Once the liquid flows across the membrane, the gold-labelled anti-IgA or cortisol will be captured by the respective s-IgA or cortisol test line resulting in the appearance of a red line (Figure 10A). Immunoglobulin A and cortisol bind to the gold-labelled anti-IgA or cortisol antibody. If IgA or cortisol is present, it will result in fewer gold particles being captured by the s-IgA or cortisol test line. Following a ten min incubation period, the LFD is placed into the LFD reader, where the relative concentrations of s-IgA or cortisol are measured within 20 sec. The test line intensity is inversely proportional to the s-IgA or cortisol present in the sample. The IPRO LFD reader will convert the line intensity into the corresponding IgA or cortisol concentrations.

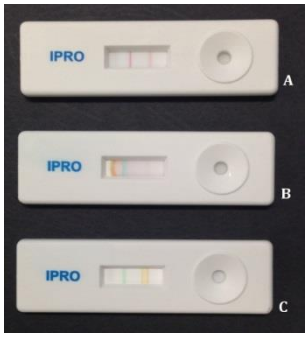

Figure 10. (A) IPRO LFD following ten minute sample incubation period; (B) IPRO LFD during ten minute sample incubation period, and; (C) IPRO LFD prior to sample incubation period.

8. 2 Saliva Flow Rate

Monitoring saliva flow rate and secretion rate is important when investigating changes in salivary analytes. Decreases in saliva flow rate as a result of dehydration or oral drying may appear to increase the concentrations of salivary analytes and several authors have proposed various methods of s-IgA expression, in an attempt to reduce misleading results. Commonly used methods of expressing s-IgA include absolute concentrations [310, 313], and ratio to total salivary protein [320, 437, 497], albumin [603] or osmolality [437, 547]. Considering the nature of the present research, in that we are manipulating the ambient temperature throughout experimental study four (Chapter Six) it was anticipated that dehydration may have an effect on saliva flow rate, potentially causing misleading results [285], as dehydration (loss of ~3% body mass) has been shown [604] to adversely affect saliva flow rate.

8. 2. 1 Calculations of Saliva Flow and Secretion Rate

Walsh et al (1999) [605] suggested the optimal way of expressing s-IgA to account for dehydration or drying effects of oral breathing throughout exercise [500, 557] is as a secretion rate, as this may be a more accurate representation of the total available IgA on the oral surface. Several authors [321, 437, 500, 605] agree the optimal equation used to express s-IgA secretion rate is flow rate × concentration (see below). To determine saliva flow rate, the length of time (min) is divided by the volume of the saliva sample (g) (see below). To determine sample volume the gravimetric method was adopted, whereby one gram of weighed saliva corresponds to one mL of saliva produced [256, 606, 607]. To express the measured concentration of the analyte, flow rate (as calculated above) is multiplied by the concentration of the sample [286, 607].

$$\text{Saliva Flow Rate (ml/min)} = \frac{\text{Volume (g)}}{\text{Time (min)}}$$

$$\text{Secretion Rate (μg/min)} = \text{Flow Rate (ml/min)} \times \text{Concentration (μg/ml)}$$

8. 3 Enzyme Linked Immuno-Sorbent Assay

The ELISA method is a sensitive technique [555] for measuring specific antibodies and antigens within a sample [608]. Antibodies are commonly manufactured in laboratory animals and purified from a blood or tissue sample for use in ELISA testing [608]. The purified animal antibodies are bound to plastic in a 96-well micro-titre plate along with a blocking agent designed to coat the plastic in the well which is not coated with the antigen [273, 608]. The primary antibody which will bind to the antigen is added to the well, followed by a secondary antibody, which will bind to the primary antigen [608]. The secondary antibody is conjugated which results in a coloured product after the substrate has been added [273, 608], which is used to identify the presence of the antigen in each well using a spectrophotometer (plate reader) [608]. Appearance of colour indicates an absence of antibodies (negative test), while the absence of colour indicates antibodies are present (positive test) [555], and there are several forms of ELISA procedures namely direct, indirect, sandwich, competitive and inhibition [555]. A competitive ELISA was used in the present thesis (Chapter Three). In competitive ELISA two specific antibodies are used; one is conjugated with enzyme and the other is present in testing sample, with both antibodies competing for the same antigen binding site [555].

A cortisol enzyme-immunoassay (EIA) kit (Salimetrics LLC, State College, PA, USA) was used in a validity and reliability study (Chapter Three) to compare with the point of care saliva analysis system used in the experimental study four (Chapter Six) in the present thesis. The ELISA used was a competitive kit, in which the cortisol in the standards and samples wells compete with cortisol conjugated to a horseradish peroxidase for the antibody binding sites on a micro-titre plate. Following an incubation period (outlined below) any unbound components are washed away, leaving the bound cortisol enzyme conjugate to be measured by the reaction of the horseradish peroxidase enzyme to the substrate TMB. Following the addition of an acidic solution which stops the reaction, the optical density (OD) was read on a standard plate reader (iMark Microplate Absorbance Reader, BioRad, California, USA) at 450nm. The amount of cortisol enzyme conjugate identified in each well is inversely proportional to the amount of cortisol in each sample.

8. 3. 1 Sample Storage

Following Salimetrics sample collection all saliva samples were weighed (Shimadzu Analytical Balance, AUW220D, Kyoto, Japan) and stored in -20°C within four hr of collection an transferred into -80°C storage after four hr for long-term storage (as per manufacturer's instructions, Salimetrics LLC, State College, PA, USA) and were only thawed on the day of analysis. All IPRO saliva samples were stored at room temperature as per manufacture guidelines (IPRO Interactive, Oxfordshire, UK) until analysis. On the day of sample analysis, frozen samples were thawed and centrifuged at 1500 × g (3000 rpm) for 15 min (Beckman Coulter Alegra x-15R Centrifuge, Brea, CA, USA) to removed mucins and other particulate matter which may interfere with antibody binding, subsequently affecting results.

8. 3. 2 Sample Collection

8. 3. 2. 1 Salimetrics Oral Swab: To standardise saliva collection procedures between point of care and ELISA testing methods, the Salimetrics SOS (Figure 11A) was used as it is similar to the IPRO OFC. The Salimetrics SOS is a polymer cylindrical swab (10mm × 30mm) and participants were instructed to place the Salimetrics SOS on top of their tongue and close their mouth, mimicking the IPRO OFC placement and procedure. After two min of saliva collection the swab was placed into SOS storage tubes (Figure 11B) and centrifuged at 1500 × g (3000 rpm) for 15 min. The saliva samples were weighed and then immediately stored in a freezer at -80°C until ELISA analysis.

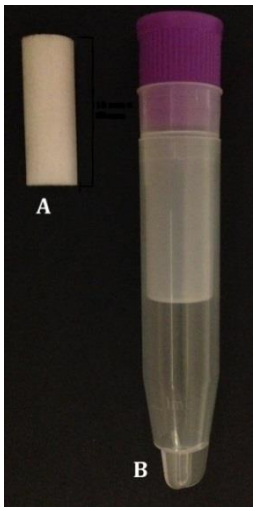

Figure 11. (A) Salimetrics Oral Swab (SOS); (B) Salimetrics SOS Storage Tube.

8. 3. 2. 2 Salimetrics Passive Drool: Salimetrics passive drool was collected in 2ml cryovials (Figure 12A) (Salimetrics LLC, State College, PA, USA) and then sealed tightly. Saliva was passed through the SalivaBio Collection Aid (SCA) (Figure 12B) into the polypropylene vial (Figure 13). Participants were encouraged to tilt their head forward throughout sample collection to promote saliva pooling in the oral cavity. Once the sample was completed they were centrifuged at 1500 × g (3000 rpm) for 15 min (Salimetrics LLC, State College, PA, USA). The saliva samples were weighed and then immediately stored in a freezer at -80°C until ELISA analysis.

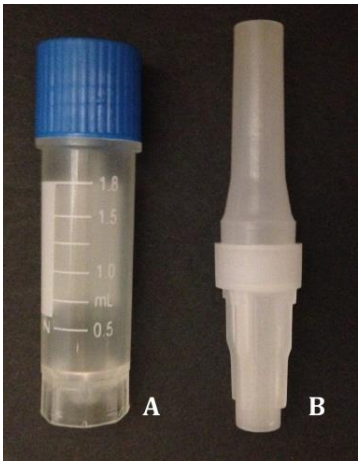

Figure 12. (A) Salimetrics Passive Drool (PD) Cryovial; (B) Salimetrics PD Saliva Collection Aid (SCA).\

Figure 13. Salimetrics PD SCA inside the Cyrovial Storage Tube.

8. 3. 3 Sample Analysis Procedure

The reagents and micro-titre plate were brought to room temperature prior to being used. The 1X buffer was prepared by diluting wash buffer concentrate (10X) with room temperature deionized water (H_2O) e.g. 100mL of wash buffer concentrate (10X) and 900 mL of deionized H_2O. Twenty five microliters (25 µL) of standards, controls and saliva samples were pipetted into the appropriate wells on the micro-titre plate, while 25 µL of assay diluent was pipetted into 2 wells to serve as the zero and two NSB wells. The enzyme conjugate was diluted by adding 15 µL to 24 mL of assay diluent. Two hundred microliters (200 µL) of the conjugate solution was added to each well using a multichannel pipette. The micro-titre plate was then placed on a plate rotator (Thermomixer comfort, Eppendorf AG, Hamburg, Germany) for five min at 500 rpm, followed by one hr of incubation at room temperature.

Following incubation at 22ºC, the micro-titre plate was washed using a squirt bottle containing 1X wash buffer prepared earlier. The plate micro-titre plate was washed four times by gently squirting the wash buffer into each well and discarding the liquid over a sink. The plate was thoroughly blotted onto paper towels before turning upright. Two hundred microliters (200 µL) of TMB substrate solution was added to each well using a multichannel pipette and the micro-titre plate was mixed on a plate rotator for five min at 500 rpm. The micro-titre plate was then incubated in the dark (covered) at room temperature for 25 min. Following incubation 50 µL of 3M stop solution was added to the micro-titre plate with a multichannel pipette and mixed on a plate rotator for three min at 500 rpm. If any green colour remained following mixing, the plate was mixed for one additional minute at 500 rpm until the green colouring had turned to yellow. The bottom of the micro-titre plate was wiped with a lint-free cloth to dry and read in a plater reader (iMark Microplate Absorbance Reader, Bio-Rad, Hercules, CA, USA) at 450nm within

ten min of adding the 3M stop solution to the micro-titre plate as per manufactures guidelines (Salimetrics LLC, State College, PA, USA).

8. 4 Core Body Temperature Monitoring

8. 4. 1 Background

Core body temperature is commonly used to monitor the effectiveness of thermoregulation [609-611], particularly throughout exercise. Traditionally there are several ways of measuring T_C namely, pulmonary artery, oesophageal, rectal and temporal measurements [612-614]. Pulmonary artery temperature is considered to be the best representation of T_C and encompasses mixed venous blood from the core and periphery [615, 616]. Despite its accuracy, pulmonary artery measurements are invasive, involving the insertion of a catheter into the right pulmonary artery [617]. Oesophageal monitoring requires a temperature probe to be placed in the lower third of the oesophagus [618], while rectal thermometers must be inserted approximately 10cm past the anal sphincter [614]. A simple and non-invasive measure of T_C is temporal monitoring, however as this involves an infrared scan of the forehead, it can be inaccurate and easily influenced by sweating [614, 619]. The ingestible temperature sensor is another method commonly used while monitoring T_C in the athletic population as it does not interfere with exercise. Ingestible temperature sensors were utilised throughout the present thesis due to their ease of use and difficulties associated with recruiting young, recreationally active female participants to consent to rectal and/or oesophageal probes.

The ingestible sensors are generally the size of a vitamin (Figure 14B), swallowed prior to physical activity and transmit temperature measurements to an external system for analysis or monitoring [614]. The ingestible sensor is a valid measure of T_C throughout walking, running and cycling [611, 620-627] when compared with oesophageal and rectal measurements and maintains its correlation with the aforementioned T_C measures during active and passive heating [628]. Additionally, the reliability of the ingestible sensor was reported with an intraclass correlation (ICC) of 0.99 (95% CI, 0.93-1.05) [625] following two bouts of 90 min shuttle running separated by seven days when compared with rectal measurements. Timing of ingestion of the sensor could have implications for the temperature readings throughout exercise. Additionally, food and beverage intake may also affect the temperature readings [614, 629] with the ingestion of cold water (5°C to 8°C) reported [629] to affect the sensor readings for up to eight hr following ingestion, however this may vary depending on the digestive speed on individuals [614]. A recent investigation [630] found it took 18.2 ±2.5 min for the ingestible sensor to leave the stomach following food intake, warranting the demand for a standardised time of ingestion in relation to

food and beverage intake surrounding acute studies [614]. If the period of sensor ingestion is too close to food intake it is likely that the temperature readings will be elevated due to the energy used to digest the food contents [614, 631].

8. 4. 2 Procedure

The CorTemp™ core body temperature monitoring system provides highly accurate temperature measurements (±0.1°C). The temperature sensor or capsule is a small electronic device (0.88 inch length × 0.42 inch diameter) encapsulated in an epoxy resin, and coated in silicone (Figure 14B). Each individual capsule was calibrated by serial number to the hand held core temperature device prior to the beginning of monitoring, to minimise variance in capsules.

The capsule was swallowed approximately eight hr prior to monitoring in experimental study four (Chapter Six) of the present thesis to ensure the sensor has reached the intestinal tract prior to monitoring, to avoid inaccurate readings due to food and beverage intake. Participants were instructed to remove the attached magnet from the capsule prior to consumption, which initialises the silver-oxide battery inside the capsule. Upon participant arrival to the exercise laboratory the handheld CorTemp™ Data Recorder (Figure 14A) begins reading the signal from the ingested sensor, which is converted from a signal frequency into digital temperature data. Throughout monitoring the handheld monitor was attached to the lower back of the participant as recommended by the manufacturer. The digital temperature data was programed to record at 20 sec intervals and the temperature data was downloaded via CorTrack™ II software to a PC for interpretation and analysis.

Figure 14. (A) CorTemp Data Recorder; (B) CorTemp Temperature Capsule.

8. 5 Long-Graded Exercise Test to Exhaustion

A long-graded cycling test to exhaustion was performed by each participant in preparation for experimental study four (Chapter Six) of the present thesis. The primary aim of the graded exercise test to exhaustion was to identify lactate threshold; the point at which lactate accumulation was greater than removal, which was used to calculate exercise intensity workloads or subsequent studies. The lactate threshold was identified as the point (exercise stage) prior to a rise in BLa concentrations of 4.0 mmol/L or greater.

8. 5. 1 Test Protocol

The long-graded exercise test to exhaustion was conducted on a cycle ergometer (Lode, Sport Excalibur, Netherlands) with an attached programmable control unit (Lode, Sport Excalibur, Netherlands). Participants began cycling at 45 watts, with 15 watt increases at four min intervals until BLa was greater than 4.0 mmol/L, as measured by a handheld automated device (Lactate Pro, ARKRAY Inc., Japan) from arterialised blood (earlobe). Upon reaching a BLa concentration of greater than 4.0mmol/L power output was increased by 15 watts every 30 sec until volitional exhaustion. Gas exchange was monitored via open circuit spirometry (COSMED, Chicago, Illinois, USA) to identify $\dot{V}O_2$ max (see calculations below, section 8.5.2) and minute ventilation (L/min). At the end of each four min stage, HR (RS800cx, Polar Electro Oy, Finland; HR), Bla

(Lactate Pro, ARKRAY Inc., Japan), RPE and blood pressure were collected. Throughout the entire test, cadence was required to be between 70-80 rpm; if cadence fell below this level the test was terminated.

8. 5. 2 $\dot{V}O_{2MAX}$ Calculations

$\dot{V}O_{2MAX}$ = Inspired Oxygen (InspO2) – Expired Oxygen (ExspO2)

Relative $\dot{V}O_2$ (ml/kg^{-1}/min^{-1}) = $\frac{\text{VO2 (ml/min)}}{\text{Body Mass (kg)}}$

Absolute $\dot{V}O_2$ (L/min) = $\frac{\text{VO2 (ml/kg}^{-1}\text{/min}^{-1}\text{)} \times \text{Body Mass (kg)}}{1000 \text{ (ml)}}$

8. 6 Threshold Trials

Lactate threshold one (LT1) was identified from the long-graded incremental cycling test to exhaustion (see section 8. 5. 1), and was used a reference point for two subsequent threshold trials. Each threshold trial comprised of 30 min of cycling (Lode, Sport Excalibur, Netherlands) at 90% of LT1 (see equation below; section 8. 6. 1), 15 min at 135% and 7.5 min at 190% of LT1. The purpose of this protocol was to allow the participant to achieve steady state in the first stage, and progressively increase exercise intensity to a highly demanding final stage. Testing protocol remained identical for both threshold trials, the only difference being ambient temperature; with one trial conducted at 22°C and one at 35°C separated by a period of two-three days.

8. 6. 1 Exercise Workloads Equations:

The three stage workloads for threshold testing is 90%, 135% and 180% of LT1 =

E.g. Assuming LT1 occurred at 90 watts then the equation is as follows;

90 × 0.9 = 81 watts

90 × 1.35 = 122 watts

90 × 1.80 = 162 watts

8. 6. 2 Procedure

Participants arrived at the exercise laboratory fasted (food and water) from 8:30pm the night before. Upon arrival participants were seated in a quiet laboratory where they provided one saliva sample for IgA and cortisol (IPRO Interactive, Wallingford, UK), resting HR (RS800cx, Polar Electro Oy, Finland), T_C (HQ Inc., Palmetto, Florida, USA), RPE and body mass (following bladder voiding). Additionally, a venous sample for estrogen and progesterone levels was collected (see section 8. 6. 2. 1 Blood Collection) while the participant was lying quietly in a supine position via EDTA blood sample tubes and measured on a Beckman Coulter counter.

Participants then entered the exercise chamber for 60 min of passive temperature acclimation. Participants were provided with a standardised breakfast of raisin toast with butter and jam (1 slice + 0.33 per 5kg over 50kg body mass) and water (5mL per/kg^{-1} body mass). Participants were instructed to finish food and water prior to 30 min of acclimation. Every 15 min throughout acclimation, HR (bpm), RPE, T_C and blood pressure were recorded and participants completed an athlete pre-test questionnaire (Appendix Five). Following 30 min of acclimation, another saliva sample was collected, followed by a subsequent venous blood sample. The participant was weighed (following bladder voiding) and prepared for the exercise trial. While seated at rest on the cycle ergometer (Lode, Sport Excalibur, Netherlands), resting HR, RPE, BLa and T_C were collected.

Following the collection of baseline measurements, the participant was instructed to begin cycling at their pre-determined workload. Several measures including HR, blood pressure and RPE were recorded every 7.5 min throughout the exercise protocol, with BLa measured at 15 min, 30 min, 45 min and 52.5 min (exercise cessation). Core body temperature was recorded at 20 second intervals throughout the entire threshold test, with values averaged for the minute surrounding each of the time points in the threshold test. Immediately following exercise, participants were instructed to stop cycling and remain seated on the bike. A saliva sample for IgA and cortisol was immediately collected, as well as a final blood pressure measurement to ensure it was safe for the participant to disembark the cycle ergometer.

8. 6. 2. 1 Blood Collection

Participants were instructed to lie supine on a collection bed in a quite exercise laboratory. The phlebotomist performing the procedure gathered EDTA tube and a supplies trolley which was positioned alongside the collection bed for convenience. A suitable site for venepuncture was selected by the phlebotomist and a tourniquet was placed approximately 3-4 inches above the selected site. Following the application of appropriate personal protective equipment, vein palpation occurs. Upon selecting a vein for venepuncture, the area was cleaned and left to dry. The participant was instructed to squeeze their hand while the phlebotomist used their thumb to taut the skin over the puncture site. The needle was inserted into the vein at a 15-30º angle. Blood was drawn into two EDTA tubes and the tourniquet was release during sample collection. Following an adequate sample, the needle was removed from the participants arm and cotton wool was immediately placed (with pressure) on the puncture site for one-to-two minutes. Following compression, as band aid was placed over the incision site. All contaminated materials were disposed of in appropriate sharps and medical waste containers. Blood samples were immediately inverted and placed on ice. Samples were transported to a centrifuge where they were spun for

ten minutes. Following centrifugation (Beckman Coulter), samples were stored in a freezer at -80º until sent for analysis at an external laboratory.

8. 7 Duel Energy X-Ray Absorptiometry (DEXA)

8. 7. 1 Background

Duel Energy X-Ray Absorptiometry (DEXA) is becoming increasingly used in research and clinical practice to identify body composition and bone mineral density [632-634], likely due to its non-invasive, ease of use and low radiation exposure [635, 636]. The radiation exposure from a GE Lunar Prodigy whole-body scanner (DEXA; Prodigy Pro, GE Healthcare Lunar, Shanghai, China) produces 2 μSv, which is less radiation than normal daily exposure [637]. Duel Energy X-Ray Absorptiometry is reportedly [637-642] accurate and precise for measuring bone mineral density and body composition.

The supine body is scanned in a rectilinear manner, segmenting the body into a series of pixels [643]. Within this series of pixels the photon attenuation is measured at two different energies, with photon attenuation assumed to be a function of tissue composition *in vivo* [643-645]. In DEXA scanning, the body is divided into three components which are considered different by their X-ray attenuation properties i.e. fat, bone mineral and fat free or lean tissue [643]. Within any pixel, the differential absorption of two photon energies of only two components can be determined [643]. Soft tissues which largely consist of water and organic compounds, reduce photon flux substantially more than bone mineral, with the presence of bone readily identified in pixels compared with those with no bone present [643]. In the pixels with no bone present, a distinction is made between fat and lean fractions within the soft tissue present [643]. In these instances the composition of these soft tissue pixels is extrapolated to the soft tissue overlying bone to identify total body fat and lean tissue [643].

8. 7. 2 Procedure

Participants were instructed to refrain from eating and drinking in the two hours prior to the DEXA scan, as this may affect the water content of their stomach, potentially causing misleading results [646-648]. Participants were asked to bladder void immediately prior to scanning due to the same principle. Prior to undertaking the DEXA scan, participants were asked to complete medical history (Appendix One), physical activity readiness (Appendix Two) and menstrual cycle / oral contraceptive history forms (Appendix Four). Additionally, following verbal and written explanations of the study, participants were asked to sign informed consent (Appendix Three). Participants removed all metal including hair clips and piercings from their body and no zips or

under-wires were present on clothing throughout the scan. Trained technical staff positioned the participant on the DEXA scanning bed, lying supine with hands and feet secured in paddles (as per guidelines developed by Nana et al 2012 and adopted by the Australian Institute of Sport [649, 650]). Participants were instructed to avoid talking or moving for the duration of the total body composition scan, as this may cause unnecessary movements in the chest cavity and triggering misleading results. The x-ray arm began to move from one end of the scanning bed to the other while identifying the relative contribution of bone mineral and soft tissue (adipose and lean) for each pixel. The x-ray scanning process takes six min and twenty sec to complete.

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APPENDICES

Appendix A. Medical History Form

Medical History Questionnaire

Name: _____

Address: _____

_____

_____

Phone: ( ) _____ (W)

Phone: ( ) _____ (H)

Email:

_____

Age:

_____

DOB:

_____

Please read the following questions very carefully. If you have any difficulty please advise the medical practitioner.

1. **Family History**. Indicate if any of your immediate family (parents, brothers, sisters, grandparents) have experienced any of the following, the age at which diagnosis occurred, and the person's relationship to you.

Relationship & Age

High Blood Pressure _____

High Cholesterol _____

Heart Disease _____

Stroke _____

Diabetes _____

Cancer

_____

2. **Your Personal Medical History**. Indicate symptoms that apply to you, by placing a cross ☒ in the box

- ☐ Pain or discomfort in chest following exercise, eating or exposure to cold
- ☐ Frequent heart palpitations or flutter
- ☐ Pain in lower lungs when walking or climbing stairs
- ☐ Unusual shortness of breath
- ☐ Very poor exercise tolerance
- ☐ Frequent dizziness
- ☐ Chronic cough
- ☐ Frequent colds or flu
- ☐ Frequent headaches
- ☐ Frequent aches or pains in joints
- ☐ Frequent backache
- ☐ Other current symptoms that exercise may affect

Are you presently experiencing, or have you ever been treated by a doctor for any of the following?

Place a cross in the box

3. **Allergies:** Hay fever, Eczema, Other rashes

☐ Yes

☐ No

Details

_____

4. **Lung Problems** (Asthma/Emphysema/Bronchitis/ Shortness or Breath/Other)

☐ Yes

☐ No

Details

_____

5. **Heart Problems** (Rheumatic Fever/Chest Pains/Palpitations/Ankle Swelling/Other)

☐ Yes

☐ No

Details

_____

6. **Blood Pressure Problems**

☐ Yes

☐ No

Details

_____

7. **Cholesterol Problems**

☐ Yes

☐ No

Details

_____

8. **Gut Problems** (Ulcer/Abdominal Pain/Diarrhoea/Constipation Hernia/Other)

☐ Yes

☐ No

Details

_____

9. **Unexplained Weight Loss**

☐ Yes

☐ No

Details

_____

10. **Urinary Problems** (Burning/Difficulty with control of urine)

☐ Yes

☐ No

Details

_____

11. **Blood Loss (**In vomit/Sputum/Bowel Action/Urine)

☐ Yes

☐ No

Details

_____

12. **Easy Bruising**

☐ Yes

☐ No

Details

_____

13. **Endocrine Problems** (Diabetes/Thyroid/Other)

☐ Yes

☐ No

Details

_____

14. **Fitting, Fainting, Blackouts, Loss of Consciousness, Muscle Weakness, Loss of Sensation**

☐ Yes

☐ No

Details

_____

15. **Headaches**

☐ Yes

☐ No

Details

_____

16. **Sight or Hearing Problems**

☐ Yes

☐ No

Details

_____

17. **Nervous Conditions**

☐ Yes

☐ No

Details

_____

18. **Bone or Joint Injury (**Back/Knee/Ankle/Hip/Shoulders)

☐ Yes

☐ No

Details

_____

19. **Other Joint Problems**

☐ Yes

☐ No

Details

_____

20. **Work Related Injuries**

☐ Yes

☐ No

Details

_____

21. **Are you exposed to a noisy/or dusty environment?**

☐ Yes

☐ No

Details

_____

22. **How often do you take over the counter medications such as aspirin, etc.?**

☐ Daily

☐ Weekly

☐ Occasionally

☐ Never

23 **Medication.** Are you taking any medication prescribed by your Doctor or other Health Care provider? If so, list details, i.e., type of drugs, dosage.

_____

_____

24 **Sleeping patterns.** How many hours do you sleep on average per night?

_____ hours.

25. **Do you ever have trouble falling asleep?**

☐ Yes

☐ No

☐ Occasionally

26. **Smoking Status**

☐ Never smoked
☐ Quit smoking more than 10 years
☐ Quit smoking less than 10 years
☐ Currently smoke (number of years _____)

27. **If currently smoking, how many cigarettes do you currently smoke per day?**

_____

28. **Physical Activity.** How many times per week do you exercise for at least 20-30 minutes continuously?

☐ Do not have a regular program
☐ Once per week
☐ 2 - 3 times per week
☐ 4 - 5 times per week
☐ More than 5 times per week

29 **Alcohol Consumption.** In the past two weeks list how many days you consumed an alcoholic beverage.

☐ Did not drink in the past 6 months
☐ Did not drink in the past two weeks
☐ 1 - 2 days
☐ 3 - 4 days
☐ 5 - 7 days
☐ 8 - 10 days
☐ 11 - 14 days

30 In the past two weeks list how many drinks on **average** you had **per day**.

☐Did not drink in the past 6 months
☐ Did not drink in the past two weeks
☐ 1 drink
☐ 2 – 3 drinks
☐ 4 – 6 drinks
☐ 7 or more drinks

Appendix B. Physical Activity Readiness Form

BOND UNIVERSITY

Faculty of Health Sciences and Medicine

PHYSICAL ACTIVITY

READINESS QUESTIONNAIRE (PAR-Q)

Please read the following questions carefully.

If the answer to the question is YES, place YES inside the box.

If the answer to the question is NO, place NO inside the box.

1. Do you suffer from any form of diabetes? ☐
1. Do you suffer from asthma or breathing difficulties? ☐
2. Has your doctor ever said you have heart trouble? ☐
3. Do you frequently suffer from pains in your chest? ☐
4. Do you often feel faint or have spells of severe dizziness? ☐
5. Has a doctor ever said your blood pressure was too high? ☐
6. Has a doctor ever told you that you have a bone or joint problem, such as arthritis, that has been aggravated by exercise, or might be made worse with exercise? ☐
7. Is there any good physical reason, not mentioned here, why you should not participate in physical activity, even if you wanted to? ☐
8. Are you between the age of 18 and 35 years? ☐

Signature of participant: …… Date / /

Witness: ……... Date / /

Appendix C. Informed Consent Form

Faculty of Health Sciences and Medicine

INFORMED CONSENT FORM

Chief Investigator(s): Name(s): Rhiannon Fisher
Faculty: Health Sciences and Medicine
Contact number(s): 0400836073
Supervisor: Assistant Professor Chris McLellan

Project Title:

The effect of oral contraception on athletic performance and several parameters of human physiology in response to exercise in varying temperatures.

(I) I have read the subject information sheet for the research project "The effect of oral contraception on athletic performance and several parameters of human physiology in response to exercise in varying temperatures" and clearly understand the content, and what is being asked of me as a volunteer in the experiment / project.

(II) The risks associated with my participation in the experiment have been clearly explained to me and I clearly understand the risks involved in my participation in the experiment / project "The effect of oral contraception on athletic performance and several parameters of human physiology in response to exercise in varying temperatures".

(III) I have been told and accept the possible benefits of my participation in the experiment / project "The effect of oral contraception on athletic performance and several parameters of human physiology in response to exercise in varying temperatures".

(IV) I have had the opportunity to ask questions about the experiment / project "The effect of oral contraception on athletic performance and several parameters of human physiology in response to exercise in varying temperatures" and the questions I have asked have been answered to my satisfaction. I also understand that I can ask questions about the experiment / project and my participation in the experiment at any time.

(V) I understand that my records will be handled in a confidential manner and that any reporting of my personal results will be anonymous or included together with the results of other participants, as an average result for the group of volunteers.

(VI) I clearly understand what is being asked of me to participate in the experiment / project and I understand the risks and benefits of my participation in the experiment / project "The effect of oral contraception on athletic performance and several parameters of human physiology in response to exercise in varying temperatures" and I agree to participate as a volunteer.

(VII) I understand that I can withdraw from the experiment / project "The effect of oral contraception on athletic performance and several parameters of human physiology in response to exercise in varying temperatures" at any time without penalty or guilt or aggravation from the investigators.

(VIII) I understand that at the appropriate time I will receive feedback on my performance in the experiment / project "The effect of oral contraception on athletic performance and several parameters of human physiology in response to exercise in varying temperatures".

(IX) I understand that the experiment / project will be carried out as described in the information statement, a copy of which I have retained. I understand that whether or not I decide to participate is my decision and will not affect my relationships with Bond University.

(X) I give my consent to participate in the experiment "The effect of oral contraception on athletic performance and several parameters of human physiology in response to exercise in varying temperatures".

Please tick the appropriate box

- ❑ The information I provide can be used by other researchers as long as my name and contact information is removed before it is given to them.
- ❑ The information I provide cannot be used by other researchers without asking me first.
- ❑ The information I provide cannot be used except for this project.

Signatures: ……. ……

Investigator(s) Date

……. ……

Participant Date

…….. ……

Witness Date

Appendix D. Exercise Pre-screening and Menstrual Cycle / Oral Contraceptive History Form

Exercise Pre-Screening Form

Name: _____ Date:_____

Baseline Measurements

Height:_____(cm)

Weight:_____(kg)

BMI: _____(kg/m^2)

Waist Girth: _____(cm)

Resting BP:_____ (mmHg)

1. **Have you ever been told you have high blood sugar? Yes / No**
2. **Have you spent time in hospital (including day admission) for any medical condition/illness/injury during the last 12 months? Yes / No**

_____

_____

_____

3. **Are you currently taking prescribed medications for any medical condition? Yes / No**

_____

_____

_____

4. **Are you pregnant or have you given birth within the last 12 months?**

Yes / No

_____

_____

_____

5. **Do you have any muscle, bone or joint pain or soreness that is made worse by particular types of activity?**

Yes / No

_____

_____

_____

_____

Menstrual Cycle and Oral Contraceptive History

Age of Menarche (First period): _____ (years)

Age of initial oral contraceptive use: _____(years)

What type of oral contraceptive pill did you use? (Please circle)

Monophasic Biphasic Triphasic Progesterone only i.e. mini pill

If known, please state the brand of pill used:

_____

How physically active have you been since menarche? (Please circle)

Sedentary Low (1-2 times/wk) Medium (3-4 times/wk) High (5+ times/wk)

Comments:_____

_____

Have you experienced any menstrual disturbances since menarche? Yes / No

If yes what disturbances have you experienced? How long did you experience them? What treatment was used?

_____

_____

_____

_____

_____

Are you currently using oral contraception? Yes / No

If yes, what type, brand and duration of use?

_____

_____

_____

_____

_____

Have you used oral contraception in the past?

Yes / No

If yes, what type, brand, duration of use and age of use?

_____

_____

_____

_____

_____

If you are taking oral contraception please complete Section A. If you are not taking oral contraception please complete Section B.

Section A

(Please complete if you are currently taking oral contraception)

Please rank in order from most important (1) to least important the reason(s) that you take oral contraception. You do not have to rank all 7, only rank those relevant to you.

_____ Birth Control

_____ Cycle Control (regular period or timing of menstruation).

_____ To reduce menstrual symptoms (please tick):

- ☐ Heavy Period
- ☐ Painful Period
- ☐ Bloating
- ☐ Breast Tenderness
- ☐ Mood Disturbance
- ☐ Other. Please Specify. _____

_____ Relief from polycystic ovary syndrome or other syndrome/disease

_____ To reduce acne

_____ Decrease risk of anaemia, or reduced bone mineral density

_____ Other. Please Specify. _____

Do you have any concerns about taking the oral contraceptive pill? Please Specify.

_____

_____

_____

If you are taking oral contraception please complete Section A. If you are not taking oral contraception please complete Section B.

Section B

(Please complete if you are not currently taking oral contraception)

Please rank in order from most important (1) to least important the reason(s) that you do not take oral contraception. You do not have to rank all 9, only rank those relevant to you.

_____ Currently using other form of birth control or hormone therapy. Please Specify.

_____

_____ Side effects (please tick):

- ☐ Weight Gain
- ☐ Increase Appetite
- ☐ Acne
- ☐ Nausea
- ☐ Headache
- ☐ Mood Change
- ☐ Reduced Libido
- ☐ Other. Please Specify. _____

_____ Medical Concerns (please tick):

- ☐ Deep vein thrombosis / blood clotting
- ☐ Increased blood pressure
- ☐ Other. Please Specify. _____

_____ Medical Condition (please tick):

- ☐ Cardiovascular Disease
- ☐ Other. Please Specify. _____

_____ Wish to become pregnant

_____ Commitment to take each day

_____ Contains hormones / not natural

_____ Reduced exercise performance

_____ Other. Please Specify. _____

Appendix E. Athlete Pre-Test Questionnaire

ATHLETE PRE-TEST QUESTIONNAIRE

Sports Scientists

Dr Clare Minahan

Griffith University Sports Science

School of Allied Health Science

GRIFFITH UNIVERISTY QLD 4222

Contact Details

Telephone: 61 (7) 5552 7842

E-mail: c.minahan@griffith.edu.au

Tester:_____ Date:_____

Sport:_____ Squad:_____

Testing Location:_____ Testing Surface:_____

Temperature:_____ Humidity:_____ Testing Time:_____

Name of athlete:_____ Date of birth:_____

Age:_____ Gender: ☐ Male ☐ Female Height (cm):_____ Mass (kg):_____

Home address:_____ Home phone:_____

Email address:_____ Mobile phone:_____

Diet

How would you rate your diet over the last 2 days? ☐ Poor ☐ OK ☐ Good ☐ Excellent

How many hours ago did you eat your last meal? _____

Recent foods eaten over the last 24 h:

_____

_____

_____

_____

Meal	Foods (including drinks)	Portion size (cups, grams)
Breakfast		
Snacks		
Lunch		
Snacks		
Dinner		

Environment

Have you been training in hot conditions in the last 2 weeks? ☐ Yes ☐ No

If yes, provide details: _____

Have you been training or sleeping at altitude in the last 2 weeks? ☐ Yes ☐ No

If yes, provide details: _____

Illness

Are you currently suffering from any type of illness? ☐ Yes ☐ No

If yes, provide details: _____

Have you had any type of illness or health problem in the last 2 weeks? ☐ Yes ☐ No

If yes, provide details: _____

Injury

Do you currently have any injuries? ☐ Yes ☐ No

If yes, provide details: _____

Have you had any injuries in the last 2 weeks? ☐ Yes ☐ No

If yes, provide details: _____

Medications and Supplements

Are you currently taking any medication? ☐ Yes ☐ No

If yes, provide details: _____

Have you taken any medication in the last 2 weeks? ☐ Yes ☐ No

If yes, provide details: _____

Have you taken any supplements in the last 2 weeks? ☐ Yes ☐ No

If yes, provide details: _____

Motivation

Evaluate your motivation for training today. ☐ Poor ☐ OK ☐ Good ☐ Excellent

Evaluate your motivation for testing today. ☐ Poor ☐ OK ☐ Good ☐ Excellent

Training

Evaluate your last week of physical training. ☐ Easy ☐ Moderate ☐ Hard ☐ Very Hard

How fatigues are you today? (0= not at all; 5 = extremely) _____

How many hours ago did you last exercise? _____

Describe your last three training sessions:

Time	Training Session	Difficulty (easy, moderate, hard)
Today		
Yesterday		
2 days ago		

Travel

Have you had to travel over the last 7 days? ☐ Yes ☐ No

If yes, provide details:

_____

_____

_____

_____

_____

_____

_____

_____

Miscellaneous

Please provide any additional information that you believe may influence your testing results.

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

Appendix F. Fisher, R. N., McLellan, C. P., & Sinclair, W. H. (2015). The Validity and Reliability for a Salivary Cortisol point of Care Test. Journal of Athletic Enhancement, 4:4. (In print form).

Fisher et al., J Athl Enhancement 2015, 4:4
http://dx.doi.org/10.4172/2324-9080.1000204

Journal of Athletic Enhancement

Research Article A SCITECHNOL JOURNAL

The Validity and Reliability for a Salivary Cortisol Point of Care Test

Fisher RN[1], McLellan CP[1] and Sinclair and WH[2]

Abstract

Introduction: Saliva analysis is commonly used in applied sport science research and practice due to convenience and ease of sample collection with traditional measures requiring immediate refrigeration and taking several hours to analyse. Therefore, the aim of the present study was to evaluate the validity and reliability of measuring salivary cortisol concentration ([sCort]) *in situ* with the Individual Profiling (IPRO) oral fluid collector (OFC) method against the salmetrics oral swab (SOS) and passive drool (PD).

Methods: Ten (N=10, male=5 and female=5) healthy, recreationally active university students volunteered to participate in the present study. Participants provided three samples in trial one (i.e. one of OFC, SOS and PD). In trial two participants repeated the sample procedure from trial one, with four participants providing duplicate OFC swabs for reliability analysis. The duplicate swabs were analysed on duplicate lateral flow devices (LFD) to test for reliability.

Results: No significant difference was found between OFC and SOS (p=0.881) and PD (p=0.145) measures, showing good agreement with no bias. Both duplicate OFC and LFD samples were not significantly different from another, with an ICC of 0.890 and 0.850 respectively.

Discussion: The present study demonstrates the IPRO method to be a valid and reliable measure of [sCort] in recreationally active individuals, indicating a useful and convenient measure for salivary cortisol testing in field environments.

Keywords

Stress; Athlete monitoring; Hormonal response

Introduction

Saliva has previously been described as a filtration of blood, in that it reflects the physiological condition of the body [1,2]. The systemic circulation and salivary ducts are separated by a thin layer of epithelial cells, thus circulation facilitates the transfer of substances in to saliva through either active carriage, diffusion through the cell membrane or via a concentration gradient [2]. The lenience of this exchange is a major reason why saliva may be ideal for diagnostic purposes in an applied sport science environment, in which venepuncture may be inconvenient or unsanitary. Cortisol is amongst the most commonly assayed biomarker in saliva and is considered to be a true reflection of serum and plasma levels in healthy adults [3-6] newborns [7], children and adolescents [8] and elderly subjects [9]. Salivary cortisol remains consistent with serum cortisol levels at rest and throughout exercise as well as upon stimulation of adrenocorticotropic and corticotropin-releasing hormones [10-12]. Very low levels of a specific biomarker and low abundant proteins can be detected in saliva samples [13-15], and therefore the risk of non-specific interference and hydrostatic interactions is potentially decreased [2]. Additionally, a sample volume of between 0.5 ml-2.0 ml is generally sufficient to elicit an accurate saliva analysis, depending on the sensitivity and reliability of the analysis system being utilised [16], and can be performed frequently, more rapidly and requires less medical training then blood collection [17-21] and therefore presenting a more attractive and athlete-friendly option for use in applied sport science research and practice [14,21-25].

Cortisol is secreted in response to intense exercise [26,27,], via hypothalamic-pituitary-adrenal axis (HPA axis) activation [21]. Cortisol is primarily catabolic in nature and when secreted causes a reduction in protein synthesis and the inflammatory process, while increasingly protein degradation and compromising several parameters of immune function [28,29]. Monitoring cortisol levels may provide the coach or sport scientist with valuable information regarding the stress response to exercise or training [21], which may assist in monitoring athlete well-being as well as adaptation to-and recovery from exercise. Over a sustained period of time high exercise-induced salivary cortisol levels may reflect overtraining [30] which can lead to a reduction in peak power, as well as isometric and concentric muscle contractions [31]. When symptoms of overtraining are appearing in an athlete, early diagnostic measures, rest and regeneration are key to recovery [32]. The availability of non-invasive and convenient measures to remotely monitor the health and well-being of an athlete may allow coaches and sport scientists to monitor the exercise-induced stress response throughout training and may be used in conjunction with medical diagnosis, for the early detection of over-training.

Traditional salivary cortisol tests can take several hours to receive a result. When investigating the cortisol response to exercise, timely feedback is crucial for the appropriate management of the athlete or active individual. An innovative point of care system for salivary diagnostics has been developed for cortisol monitoring and can provide feedback within 15 min of sample collection. This point of care saliva analysis system is known as Individual Profiling (IPRO) and has the potential to drastically change the way coaches and athletes can monitor the stress response to training and competition. The IPRO point of care salivary cortisol test has undergone preliminary validity and reliability investigations in English Football League Championship Academy soccer players [33]. A total of 29 saliva samples were measured by the IPRO and were also analysed by Enzyme-Linked Immunosorbent Assay (ELISA) within 4 hr of sample collection. All samples were reported [33] to be within the measurement range for both IPRO and ELISA analysis ranging between 1.09-9.45 ng/ml and 0.50-6.26 ng/ml respectively. In addition, 16 IPRO samples were measured on two separate lateral flow devices (LFD) on two occasions, each 30 minutes apart, reporting [33] good agreement with mean within CV between repeats of 6.88%.

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Similar results have been produced for a point of care test on salivary immunoglobulin A (s-IgA) for the IPRO system [34,35].

The aim of the present study is to investigate the validity and reliability of the IPRO point of care salivary cortisol concentration ([sCort]) test against gold-standard ELISA methods in healthy, active individuals.

Methods

Participants

Ten (N=10, male=5 and female=5) healthy, active university students volunteered to participate in the present study. Participants were Exercise and Sport Science students from the same cohort, who participated in weekly recreational exercise such as running, cycling, swimming and team sports. Participants were instructed to refrain from strenuous exercise 12 hr prior to saliva collection on both testing occasions. Participants were also instructed to avoid consuming food or brushing their teeth in the 2 hr prior to sample collection and had not consumed alcohol for at least 24 hr. Participants were asked to keep their morning routines as similar as possible on each day of sample collection, with testing occurring at the same time and location on both testing occasions. All participants signed informed consent prior to participating in this study. This study was approved by the Bond University Human Research Ethics Committee (BUHREC).

Sample collection

Unstimulated whole saliva was collected by IPRO oral fluid collector (OFC) (LFD; IPRO Interactive, Wallingford, UK), Salimetrics oral swab (SOS; Salimetrics LLC, State College, PA, USA) and Salimetrics passive drool (PD; Salimetrics LLC, State College, PA, USA) and assessed for salivary cortisol concentration ([sCort]). Participants were seated in a quiet, safe environment for saliva collection and asked to rinse their mouth out with water 10 min prior to sample collection. Saliva collection occurred on the same day and time, on two occasions separated by four weeks. Participants were considered to be under the same physiological and psychological stress surrounding both testing occasions, however it is important to note that perceived physical and psychological stress was not measured through perceived stress scales which take into consideration recent life events, chronic difficulties, trait anger and depression [36]; all factors which may influence cortisol secretion at rest, and poses a limitation of the present study. During trial one (T1), a total of three saliva samples were collected (i.e. one of each OFC, SOS and PD, in that order) (Figure 1). During the second trial (T2) the same measures were collected, in order from T1, however two OFC's were placed in the oral cavity simultaneously (OFC_1 and OFC_2) in four randomly selected participants (Figure 1). The additional OFC samples collected in T2 were analysed on two separate lateral flow devices (LFD_1 and LFD_2) (Figure 1) and compared to investigate the reliability of both the OFC swab as well as the LFD using the IPRO method.

Salivary collection and analysis procedures

Oral fluid collector (OFC): Participants placed one OFC swab on top of their tongue and closed their mouth in T1 with two OFC swabs placed on the tongue in T2 (in four randomly selected participants only). The device consists of a synthetic polymer-based swab material attached to a volume adequacy indicator stem and a dropper bottle with extraction buffer. When 0.5mL of saliva has been absorbed by the OFC, an indicator line of the swab stem turns bright blue at which point the OFC is removed from the participant's mouth and placed (swab down) into a dropper bottle. The dropper bottle contains 3mL of buffer which consists of sodium phosphate, salts, detergents and preservatives to attenuate the growth of microorganisms, as well as extraction agents to target analytes from the cotton based swab. Saliva samples are stable at room temperature and were stored according to manufacturer's recommendations (IPRO Interactive, Wallingford, UK).

For analysis, the dropper bottle containing 3mL of buffer and the OFC saliva sample was repeatedly inverted with care lightly shaken for 2 min. Two drops of the buffer solution and saliva sample mixture was placed onto the sample pad of the LFD and incubated at room temperature for 10 min, as per manufacturer's instructions (IPRO Interactive, Wallingford, UK). The liquid uses a capillary action to travel through the conjugate pad hydrating the dried conjugate. The liquid continues to flow through the nitrocellulose membrane towards the wicking pad at the end of the strip. Once the liquid flows across the membrane, the gold-labelled anti-cortisol will be captured by the cortisol test line resulting in the appearance of a red line. Cortisol binds to the gold-labelled anti-cortisol molecule; and if cortisol is present, it will result in fewer gold particles being captured by the cortisol test line. The test line intensity is inversely proportional to the cortisol concentration present in the sample. After 10 min of incubation, the LFD was placed into the Lateral Flow Reader and analysed which then converted the line intensity into the corresponding [sCort].

Salimetrics oral swab (SOS): To standardise saliva collection procedures between point of care and ELISA testing methods, the SOS was used as it is similar to the OFC. The SOS is a polymer cylindrical swab (10 mm×30 mm). Participants were instructed to place the SOS on top of their tongue and close their mouth, mimicking the OFC placement and procedure. After 2 min of saliva collection the swab was placed into a storage tube and centrifuged at 1500×g (3000 rpm) for 15 min as per manufacturers guidelines (Salimetrics LLC, State College, PA, USA). The saliva samples were weighed and then immediately stored in a freezer at -20°C until ELISA analysis.

Salimetrics passive drool (PD): PD was collected in 2 ml cryovials (Salimetrics LLC, State College, PA, USA) and then sealed tightly. Saliva was passed through the SalivaBio collection aid into the polypropylene vial. Participants were encouraged to tilt their head forward throughout sample collection to promote saliva pooling in the oral cavity. Following collection all samples were centrifuged at 1500×g (3000 rpm) for 15 min. The saliva samples were weighed and then immediately stored in a freezer at -20°C until ELISA analysis.

For analysis of SOS and PD, samples were thawed completely, placed on the vortex and centrifuged at 1500×g (3000 rpm) for 15 min as per manufacturer's guidelines (Salimetrics LLC, State College, PA, USA). All saliva samples, reagents and the assay plate were at room temperature, prior to analysis. Wash buffer concentrate of 100 mL was added to 900 mL of deionised water at room temperature for dilution. Reagents were prepared and the assay plate layout was determined, testing all samples in duplicate. Subsequently, 24 mL of diluted assay was then pipetted into the disposable tube, followed by 25 µL of assay diluent into two wells each to serve as the zero and non-specific binding wells. A further 25 µL of standards and saliva samples were added into the appropriate wells. The enzyme conjugate was diluted 1:1600 by combining 15 µL of conjugate to the 24 mL tube of

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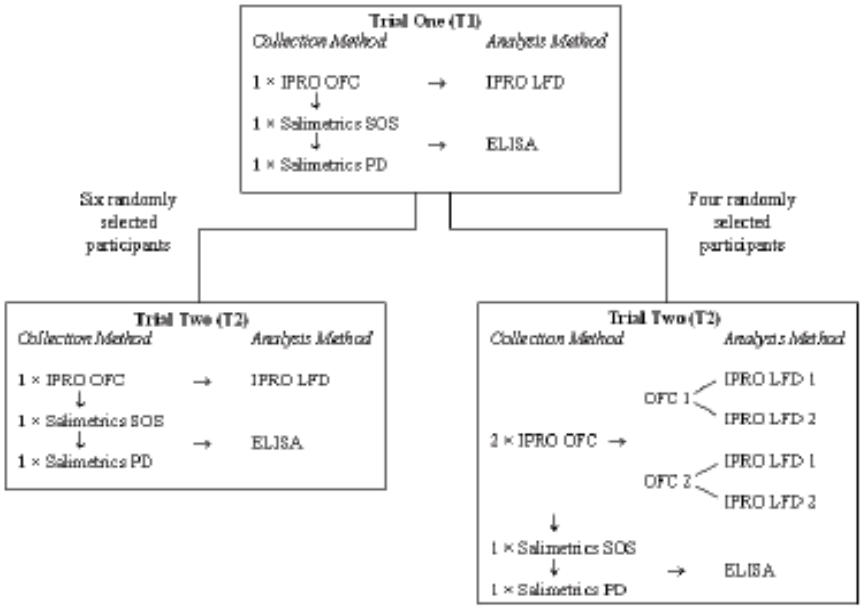

Figure 1: Schematic of sample collection procedures in trial one (T1) and trial two (T2).

diluted assay, which was placed on the vortex prior to adding 200 µL to each well using a multichannel pipette. The assay plate was mixed on a plate rotator (SpectraMax 190, Molecular Devices, Fullerton CA, USA) for 5 min at 500 rpm and incubated at room temperature for 1 hr. Following incubation, the plate was washed four times with 1X buffer, with the plate thoroughly blotted on paper towels after each wash, prior to being turned upright. Following washing, 200 µL of tetramethylbenzidine (TMB) substrate solution was added to each well with a multichannel pipette. The assay plate was the mixed on a plate rotator or 5 min at 500 rpm and incubated in the dark at room temperature for 25 min. Following incubation, 50 µL of a 3M stop solution was added to each well with a multichannel pipette. The assay plate was mixed on a plate rotator for a further 3 min at 500 rpm, or until all wells turned yellow. The assay plate was then read in a plate reader at 450 nm, within 10 min of adding the 3M stop solution.

Statistical analysis

Data was analysed for assumptions of normality using a Shapirio-Wilk test, and in the event data was violated non-parametric Friedman two-way ANOVA or Wilcoxon Signed Rank tests were conducted. A Paired t-test was completed to compare $IPRO_{T1}$ and $IPRO_{T2}$, as well as $IPRO_1$ and $IPRO_2$ and LFD_1 and LFD_2 samples for intra-tester and OFC and LFD reliability, with interclass coefficient (ICC) ± SEM presented. Pearson's correlation coefficient and One-way Random model for intra-rater reliability were completed to confirm reliability. A Bland-Altman graph was used to assess the level of agreement between IPRO compared with SOS and PD with at 95% CI reported. Alpha intervals were set at $p=<0.05$ for all correlation and hypothesis tests with results presented as mean ± SD. Statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS version 20, Chicago, IL, USA).

Results

Validity

A Friedman two-way ANOVA did not identify any significant ($p=0.819$) difference between OFC and SOS or PD. A follow up analysis of pair wise comparisons was completed in the form of a Wilcoxon signed rank test, revealing OFC was not significantly different from SOS ($p=0.881$, $r=-0.33$) or PD ($p=0.145$, $r=0.32$) measures. The level of agreement between IPRO vs. SOS (Figure 2) and IPRO vs. PD (Figure 3) were assessed via a Bland-Altman graph, revealing unbiased agreement between OFC and both SOS and PD methods. Validity and reliability statistics for OFC, SOS and PD are presented in Table 1.

Reliability

Tests of normality showed no violation and Paired *t*-tests were conducted to establish reliability. No significant differences ($p=0.223$, $ICC=0.685$; p=0.02 ± 0.7 SEM) were observed between OFC_{T1} and OFC_{T2}, indicating good inter-tester reliability. Additionally, no significant difference was found between OFC_1 (6.26 ± 2.84 nmol/L) and OFC_2 (6.46 ± 3.38 nmol/L) or LFD_1 (6.35 ± 3.01 nmol/L) and LFD_2 (6.37 ± 3.00 nmol/L). Indicating good reliability of both the OFC swab ($p=0.813$, ICC 0.890; p=0.11 ± 0.4 SEM) and LFD ($p=0.977$, ICC = 0.850; $p=0.00$ ± 0.5 SEM). Descriptive statistics for OFC, SOS and PD are shown in Table 2.

Discussion

The results of the present study found the IPRO point of care test for salivary cortisol concentration [sCort] to have good agreement with the industry gold standard ELISA method. Additionally, the present study identified the OFC method of saliva collection to be more closely correlated with the SOS method ($p=0.881$) compared

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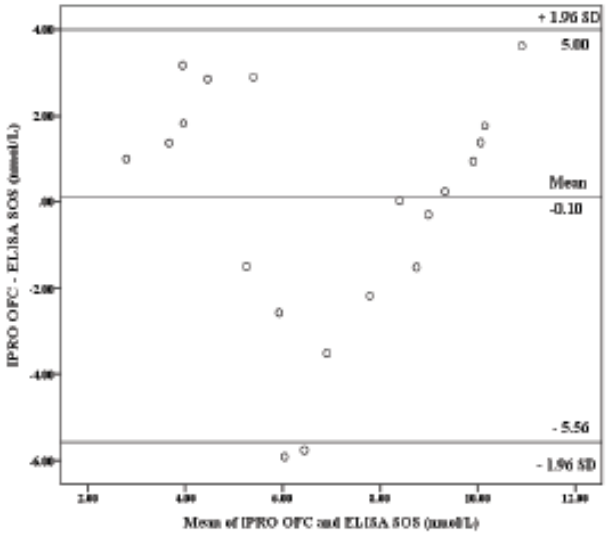

Figure 2: Bland-Altman scatter plot, agreement between Individual Profiling (IPRO) oral fluid collector (OFC) and ELISA Salimetrics oral swab (SOS). Solid lines indicate mean difference (-0.10 nmol/L), and ± 1.96 SD (5.00 nmol/L, -5.56 nmol/L). No proportional bias was present (p=0.976).

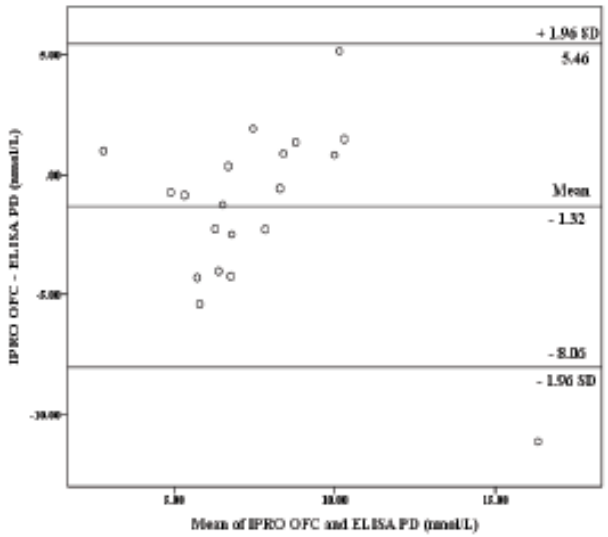

Figure 3: Bland-Altman scatter plot, agreement between Individual Profiling (IPRO) oral fluid collector (OFC) and ELISA passive drool (PD). Solid lines indicate mean difference (-1.32 nmol/L), and ± 1.96 SD (5.46 nmol/L, -8.06 nmol/L). No proportional bias was present (p=0.281). One outlier was identified.

with PD method (p=0.145) for [sCort]. This is an important finding, and suggests future salivary cortisol comparisons should preferentially compare OFC results to SOS results when necessary. This finding is in-line with previous research [37] reporting differences in results in Salivary Immunoglobulin A concentrations in cotton swab collection compared with passive drool collection and demonstrates the importance of standardising saliva collection techniques in research and practice. Both OFC (p=0.813) and LFD (p=0.977) duplicate samples were not statistically different, indicating good reliability between samples. This finding suggests the IPRO point of care method is useful for salivary cortisol testing in recreationally active individuals and investigators can be satisfied that any substantial inter-subject changes to [sCort] are not due to tester errors if proper testing protocol is followed. The results of the present study are in agreement with previous investigations in English Football League Championship Academy soccer players [33] finding the IPRO method to be in good agreement with the ELISA method for determining [sCort]. Together, these findings suggest the IPRO method of measuring salivary cortisol (via OFC collection and LFD analysis) is useful for coaches and researchers when monitoring salivary cortisol in both recreationally active individuals and elite level athletes.

Saliva analysis is commonplace in applied sport science research due to convenience of sample collection with between 0.5-2.0ml generally sufficient to elicit an accurate result [14,21-25], however sample analysis can be time consuming. The IPRO method produces results within 15 min of sample preparation, providing timely feedback, and therefore offering an appealing alternative method of stress monitoring in real-time. In addition, the IPRO LFD reader is portable and can be used in the field for real-time analysis, bridging the gap between laboratory and field testing in applied sport science research and practice. The immediate availability of information regarding physiological demands of exercise is crucial in athlete monitoring. Coaches using the IPRO method for [sCort] analysis can readily monitor the stress response of their athletes throughout training and competition, gaining specific feedback about the demands of sport and exercise, providing advantage over traditional salivary cortisol measures i.e. ELISA.

The results of the present study demonstrate the validity

Table 1: Validity and Reliability statistics for Individual Profiling (IPRO) oral fluid collector. (OFC), ELISA Salimetrics oral swab (SOS) and ELISA Salimetrics passive drool (PD) for salivary cortisol concentration [sCort].

	Value	95% upper limit	95% lower limit
Validity			
IPRO OFC vs. ELISA SOS			
[sCort] Mean Difference (nmol/L)	-0.10 ± 2.79	1.20	-1.41
Pearsons's Correlation Coefficient (r)	0.52		
IPRO OFC vs. ELISA PD			
[sCort] Mean Difference (nmol/L)	-1.32 ±3.45	0.28	-2.94
Pearsons's Correlation Coefficient (r)	0.45		
Reliability			
IPRO OFC_{T1} vs. OFC_{T2}			
[sCort] Mean Difference (nmol/L)	-0.93 ±2.26	0.68	-2.55
Intra Class Correlation (ICC)	0.658	0.901	0.125
Pearsons's Correlation Coefficient (r)	0.68		
IPRO OFC_1 vs. OFC_2			
[sCort] Mean Difference (nmol/L)	-0.20 ±1.55	-2.67	2.27
Intra Class Correlation (ICC)	0.904	0.993	0.331
Pearsons's Correlation Coefficient (r)	0.89		
IPRO LFD_1 vs. LFD_2			
[sCort] Mean Difference (nmol/L)	-0.01 ±1.64	-1.39	1.35
Intra Class Correlation (ICC)	0.868	0.971	0.514
Pearsons's Correlation Coefficient (r)	0.85		

Table 2: Descriptive statistics for Individual Profiling (IPRO) oral fluid collector (OFC), ELISA Salimetrics oral swab (SOS) and ELISA Salimetrics passive drool (PD) for salivary cortisol concentration [sCort].

	OFC	SOS	PD
[sCort] Mean ± SD (nmol/L)	6.90 ± 2.88	7.00 ± 2.86	8.23 ± 3.63
[sCort] Mean 95% CI - Lower	5.55	5.66	6.53
[sCort] Mean 95% CI - Upper	8.25	8.34	9.93
[sCort] Minimum	3.08	2.29	2.28
[sCort] Maximum	12.72	9.50	21.90

and reliability of a point of care salivary cortisol test for use in recreationally active individuals. Both the IPRO saliva collection (OFC) and analysis (LFD) aspects of the method were shown to be valid and reliable for salivary cortisol monitoring of [sCort] compared with the ELISA technique. Further reliability and validity testing should be conducted for the IPRO salivary cortisol method to confirm the findings of the present paper in a larger and more applied sample population including elite athletes.

Practical Applications

- The Individual Profiling (IPRO) method showed a good agreement with the ELISA method for salivary cortisol analysis.
- Coaches may use the Individual Profiling (IPRO) salivary cortisol method for *in situ* monitoring of the stress response to training and competition.
- The Individual Profiling (IPRO) oral fluid collector (OFC) and lateral flow device (LFD) show good reliability between samples.

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Appendix G. Fisher, R. N., Sinclair, W. H., Lovell, D. I., & McLellan, C. P. (2015). The Immunological Response to Surf Life Saving Competition. Journal of Australian Strength and Conditioning. 23(2), 15-20 (In print form).

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Original Scientific Research Study

THE RESPONSE OF SALIVARY IMMUNOGLOBULIN A TO ELITE SURF LIFESAVING COMPETITION

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BLUF

This study found elite Surf Life Saving (SLS) athletes experience non-significant increases in sIgA concentration 30 minutes post-competition in both genders and male SLS finding athletes experience a significant increase in salivary immunoglobulin A in males 61 hours post competition.

ABSTRACT

The present study examined the response of salivary immunoglobulin A (sIgA) to endurance surf lifesaving (SLS) competition. Elite SLS men (n = 10) and women (n =8) volunteered to participate in the present study. Saliva samples of 0.5 mL were collected daily commencing 54 h pre-event to 61 h post-event. Saliva samples were assessed using a portable system. Saliva samples were collected at rest and prior to physical exertion or activity at approximately 0500 hours to determine the pre-and post-competition baseline values, while the post-event sample collection occurred within 30 min of race completion for each athlete, between approximately 1300 – 1600 hours.

A significant ($p < 0.05$) increase in sIgA concentration was observed 61 h post-event (49.21 ± 8.8 µl/mL) in males compared to all other time-point samples. The results of the present study indicate that there is a non-significant increase in sIgA concentration for both genders immediately following an endurance SLS event, lasting approximately 90 min. The mechanism responsible for the non-significant increases in sIgA concentrations observed post-event in both genders and significant increases in sIgA concentrations observed 61 h post event in males remains unclear and may reflect an immune-compensatory effect to protect the athlete from the acquisition of infection. Surf lifesaving athletes and coaches should monitor sIgA concentrations throughout major competition periods to observe fluctuations in mucosal immunity, which may reflect a predisposition to infection, and tailor exercise and recovery prescription accordingly to minimise the risk of infection and subsequent declines in performance.

Key Words - Respiratory tract infection, athletic performance, immunity, exercise.

INTRODUCTION

The influence of exercise on immune system function has been investigated in elite sports including swimming (12, 35) kayaking (9, 23) and running (30, 32). In particular, upper respiratory tract infections (URTI) have been identified (26) as the most common infection experienced by highly trained athletes and are frequently responsible for athlete absence from training and competition (29). Pyne (34) concluded that acquiring an URTI during training or competition may lead to detrimental effects on the health and performance of elite athletes.

Tomasi and colleagues (40) reported that a predisposition to URTI often observed in athletes may be a result of a depressed immune response following intense exercise. In particular, decreases in salivary immunoglobulin A (sIgA), the most abundant immunoglobulin at the mucosal surfaces of the upper respiratory tract, have been shown (4) to initiate up to 95 percent of all infections. Salivary immunoglobulin A represents the first line of defence against pathogens by preventing colonization and replication of viruses and bacteria on the mucosal surface of the upper respiratory tract (3). It has been demonstrated (4, 12) that decreasing concentrations of sIgA are associated with a predisposition to respiratory illness and therefore can be a useful marker for respiratory infection risk (28). Saliva analysis has become a widely accepted and utilised measure in applied research and team sport monitoring strategies (7, 23, 35) due to the non-invasive nature of sample collection and improved analysis methodologies for determining the sIgA concentration (10). The sIgA concentration has been shown (21) to correlate more highly with URTI than serum antibodies or other immune parameters and as such provides a worthwhile measure for integrated athlete monitoring protocols in individual and team sports.

Multiple studies (10, 11, 23) have investigated the effects of endurance exercise similar to that undertaken by elite surf lifesaving (SLS) athletes, reporting decreases in sIgA concentration in response to intense kayaking (9, 23) (>60% $VO2_{max}$), a seven month swim training program (12) and marathon running (30). However, other studies (17, 25, 35) have reported no change in sIgA concentration following intermittent and continuous exercise, including soccer and

rugby competition and prolonged (>1.5 hr in duration) swimming. Increased sIgA concentration following cycling (18) and basketball competition and training (39) have been reported to further demonstrate the acute impact of exercise on the immune system. Mackinnon (20) identified that the degree of sIgA suppression is dependent on the intensity of the exercise. Currently, a discrepancy exists with respect to the acute sIgA response to exercise and may be associated with methodological differences related to sample size and method of collection.

Despite current research (9, 29, 34) examining the immune response to intense training for specific sports, few studies (17, 39) have investigated the changes in sIgA concentrations during competitive exercise performance and the acute response of sIgA to elite SLS competition is unknown. Elite SLS competition is a physically demanding sport that requires substantial physical conditioning, with athletes regularly participating in multiple daily training sessions - six days a week. In SLS, the 'ironman' race has emerged as the most demanding multi-disciplinary event through the consecutive performance of the four major skills of SLS namely soft sand beach running, surf swimming, surf board paddling and surf ski paddling (38) in a single event. Mulit-disciplinary surf athletes complete each skill over varying course formats and distances and are associated with performance durations from fifteen minutes (min) to four and half hours (hr) (31) for any one event. It is also common for athletes to compete in heats, semi-finals and finals on a single day of competition (38). Despite the professional status of the sport, currently few studies (38) have investigated the effects of training and competition on the elite SLS athlete and there is a lack of understanding regarding the endocrine and immunological response of elite SLS athletes to training and competition. The aim of the present study was to investigate the sIgA response of elite men and women to a single, endurance SLS competitive event. The endurance SLS event under investigation involved continuous loops of a competitive course, which integrated surf swimming, surf board paddling and surf ski paddling in the open ocean. Male competitors raced for approximately 90 min and female athletes competed for approximately 60 min. No previous studies have examined the relationship between the exercise-related response of sIgA and the predisposition of athletes to develop URTI in SLS athletes following competition.

METHODS

Approach to the Problem: Athletes provided daily unstimulated saliva samples in a non-fasted state over a period of six days at approximately from 54 h pre-event to 61 h post-event. All saliva samples collected at training sessions occurred at approximately 0500 hours. Saliva samples collected on the day of event varied in response to the schedules time of competition; pre-event samples were collected prior to warm up between 0900 and 1100 hours, while post-event samples were collected between 1300 and 1500 hours, after cool down. The daily training and saliva collection schedule is outlined in Table 1. Data were examined for each subject at each saliva sample collection time point. Throughout the pre and post-event sample collection period, subjects participated in all scheduled training and recovery sessions (Table 1).

Table 1 - The daily training and saliva collection schedule.

Test	54hr Pre-Event	30hr Pre-Event	Pre-Event	30min Post-Event	17hr Post-Event	37hr Post-Event	61hr Post-Event
Training Schedule	0500 Swim	0500 Swim	0900 – 11:00 Pre Event	1300-16:00 Post-Event	0900 Recovery	0500 Swim	0500 Swim
Saliva Test	15min Prior to Training	15min Prior to Training	15min Prior to Warm Up	After cool down / recovery.	15min Post-Awakening	15min Prior to Training	15min Prior to Training

Subjects: A total of 25 athletes competing in the 2012/2013 Kellogg's Nutri-Grain Ironman and Telstra Ironwoman series were invited to participate in this study. Eighteen (n=18) participants (male n=10 female n=8) volunteered to participate in this study (age: 24.4 y ± 7.5 y). All athletes that volunteered to participate in the study provided written informed consent and completed medical history and physical activity readiness questionnaires (PARQ). The protocol associated with the investigation was approved by the Bond University Human Research Ethics Committee (BUHREC).

Procedures: The present study was undertaken during one of the 2012/2013 Kellogg's Nutri-Grain Ironman and Telstra Ironwoman events which was approximately 90 min in duration and included all four skills of Ironman and Ironwoman competition; soft sand beach running, surf swimming, surf board paddling and surf ski paddling that constituted separate stages of a single competition race. Each lap was a total distance of 1993m with the surf board paddling, surf swim, surf ski and run stages set at 662 m, 421 m, 750 m and 160 m respectively. Each skill was completed in sequence and repeated four times.

Unstimulated saliva was collected via an Oral Fluid Collector (IPRO OFC, Ipro Interactive, Oxfordshire, UK) consisting of a synthetic polymer based material on a polypropylene tube. The OFC has a volume adequacy indicator, providing a clear colour change when 0.5mL (± 20%) of passive saliva is collected. All subjects were instructed to swallow any saliva present, before placing the OFC on their tongue. All subjects were requested to avoid the ingestion of food and fluids other than water in the 60 minutes before providing each saliva sample and to refrain from brushing their teeth 2 hours before each saliva sample collection session. Subjects were instructed to wait for a period of 10 minutes after

their last consumption of water before commencing the saliva sample collection process. Saliva samples were collected from each athlete at their training and race venue as determined by the sample collection protocol to minimise disruption to the participants' pre-event routine and preparation.

Saliva Immunoglobulin A (µg/mL) was analysed in duplicate via a commercially available lateral flow immunoassay test kits (IPRO Lateral Flow Device (LFD), (IPRO Interactive Ltd., Oxfordshire, England) and are reported as absolute measures for within individual variation. The lateral flow principle of saliva analysis, or immunochromatographic strip (ICS) test was conducted in accordance with manufacturer instructions. For the LFD, two drops of saliva/buffer mix are added to the sample window of the LFD cassette. The liquid runs the length of the test strip via capillary action creating a control and test line visible in the test window. Five minutes after the sample is added the test line intensity is measured in an IPRO plate reader (IPRO Interactive Ltd., Oxfordshire, England). The test line intensity is inversely proportional to the sIgA concentration in the sample. The sIgA intra-assay coefficient of variation as a percentage was 8.0%.

Statistical Analysis: All data are expressed as mean ± SEM. A one-way analysis of variance (ANOVA) was used to compare differences in salivary IgA concentration for each saliva sample collection time point. When the ANOVA showed significant main effects, a Tukey's *post-hoc* test was used to identify the source of the differences. The statistical software package SPSS version 21.0 was used for all data analysis.

RESULTS

The mean baseline measures collected at 54 h, 30 h prior to and the morning of the event were 33.55 ± 5.6 µg/mL, 38.60 ± 8.8 µg/mL and 37.41 ± 8.7 µg/mL respectively for males, and 31.00 ± 6.2 µg/mL, 39.76 ± 8.4 µg/mL and 34.04 ± 7.2 µg/mL respectively, for females. A non-significant increase in sIgA concentration was observed for both genders, post-event compared to pre-event with a measure of 44.34 ± 8.4 µg/mL for males and 38.90 ± 9.3 µg/mL for females. Samples collected 17 h and 37 h post-event were measured at 39.77 ± 8.9 µg/mL and 39.85 ± 7.9 µg/mL respectively for males and 32.40 ± 6.7 µg/mL and 39.88 ± 8.6 µg/mL respectively for females. No significant change was observed for females 61 h post-event with a mean measure of 35.63 ± 7.5 µg/mL, however a significant increase ($P < 0.05$) in sIgA concentration was observed at 61 h post-event in males, compared to all other time-points, with a mean measure of 49.21 ± 8.8 µg/mL. The mean values (SEM) for both genders are shown in Figure 1.

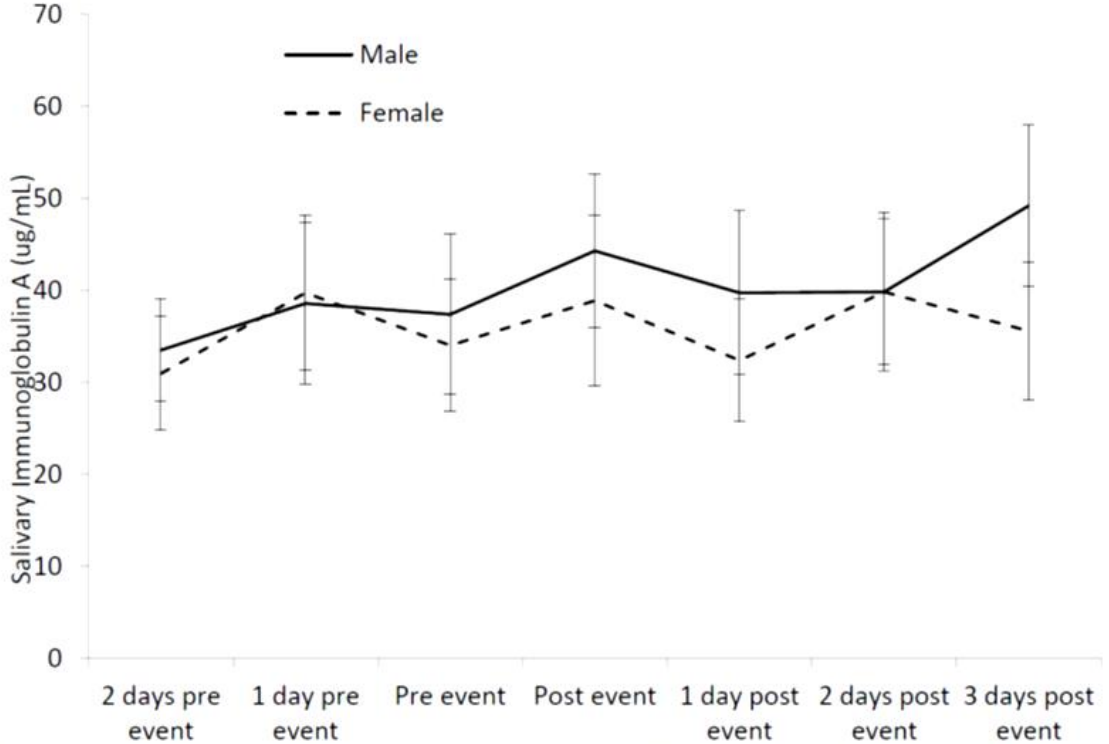

Figure 1 - Changes in salivary IgA concentration before and after a single, endurance Surf Life Saving Iron-person event of approximately 90min duration. Values are means ± SEM. * significantly ($p < 0.05$) less than 61 hr post event.

DISCUSSION

The results of the present study provide new insight into the sIgA response to elite SLS competition and demonstrate the unique characteristics of SLS in comparison to research (32) that has reported a decrease in sIgA concentration in response to high intensity swimming (12) kayaking (23) and marathon running (30). The results of the present study demonstrate that elite male and females SLS athletes experience a non-significant increase in sIgA concentration within 30 minutes post SLS endurance competition. Additionally, male athletes experience significant ($P < 0.05$) increases in

sIgA concentration 61 hr post-competition of approximately 90 min duration, when compared with all other male time-points.

The mechanism responsible for the changes in sIgA concentration during the present study is unclear, however multiple studies (14, 20) have suggested a link between physical stress from intense exercise, life style factors and annual training millage and fluctuations in sIgA concentrations. A possible mechanism associated with the results of the present study is the influence of the stimulation of the Hypothalamic-Pituitary-Adrenal (HPA) axis in response to intense exercise, which up-regulates the production of polymeric-IgA receptors and facilitates the active transport of the polymeric-IgA complex across epithelial cells (19) leading to increases in sIgA concentrations. Upon stimulation, the nervous system has been shown to increase the secretion of sIgA into saliva through the stimulation of the sympathetic and parasympathetic nervous systems (24, 33). The autonomic nervous system supply's nerves to the immune cells in lymphoid organs, and has been shown to induce changes in local immune processing (2). In particular, the autonomic nervous system has been shown to enhance the transfer of sIgA (37). The SLS event examined in the present study required athletes to physically exert themselves to exhaustion over approximately 90 min in attempt to be victorious and accumulate sufficient points in the series to guarantee automatic qualification for the subsequent competition. Combining the additional pressure of concerns over requalification for the following year with the challenging oceanic conditions of this particular single, endurance SLS event, it is therefore possible that physical exhaustion and psychological stress may have contributed to amplified HPA axis activation, subsequently increasing sIgA concentrations as observed at 61 h post-event in males, as an immune-compensatory effect.

Mackinnon (23) suggested intense daily kayaking (>70% maximal heart rate) in elite athletes may result in adverse changes in the production and secretion of sIgA. In particular, a malfunction of the mucosal plasma cells, which migrate from the gut to the minor salivary glands, which is the major source of IgA in whole saliva (23). The adverse changes in the production and secretion of sIgA may occur in response to variations in temperature in mucous membranes or from decreases in nasal fluids, ultimately leading to a reduction in IgA output (40). Elite SLS athletes undergo substantial physical conditioning to prepare for competition, with athletes commonly training for several years before achieving optimal strength and endurance to successfully compete at the elite level. It is possible that a cumulative effect of training history may have contributed to the changes observed in the present study.

Salivary IgA concentrations were not altered in male and female elite swimmers in response to a five month period of training leading into the 1998 Commonwealth games (35). Conversely, intense training in a cohort of 26 elite swimmers over a five month period was directly linked to a suppression of mucosal immunity (11). Similar results were observed over a three week training period in elite kayakers, showing decreases in sIgA concentration (23). The distinction between the results of the present study and studies that examined swimming (11, 35) and kayaking (23) are likely to be associated with variation in training volumes, competitive distances, sample collection times and the unique nature of multi-event endurance sports such as SLS. The athletes in the present sudy were competing until exhaustion in a crucial event, it is therefore difficult to compare to other studies (11, 35) which investigated training sessions, where it is possible that the same exercise intensity as experienced in a competition setting, was not obtained in these studies. Allgrove et al (1) investigated cycling to exhaustion, reporting very-high intensity exercise was sufficient to induce increased transportation of sIgA into the saliva, immediately following exercise, as a result of sympathetic stimulation. Allgrove (1) suggested it is possible for exercise to exhaustion to activate the sympathetic nervous system and cause an increase in sIgA concentration. Previous studies (8, 22) have concluded exercise induced changes in sIgA concentrations return to resting levels within 24 hours of exercise cessation. It is possible that increased neural activation throughout the competition and upon the resumption of regular training at approximately 37-61 h post-event, may have provoked sympathetic nervous system activation and subsequent increases in sIgA concentrations. The increases in sIgA concentartions observed post-event in both genders and 61 h post-event in males may have been exacerbated by below normal baseline measures. It is possible that baseline measures were lower than average as particpants were in a taper period of reduced training volume in preparation for the event, and therefore the reductions in training volume may not have been sufficient to elicit a charcacteristic change in sIgA concentrations in the days prior to the event.

The effect of gender on sIgA has been investigated in the athletic population concluding females often exhibit slower whole saliva secretion rates than males (5) and consequently higher sIgA concentration than males (16). The slower whole saliva secretion rate commonly observed in females may be due to hormonal differences associated with gender or the use of oral contraceptive medications (16). The menstrual cycle and oral contraceptive medications have been shown to influence the free cortisol response, with lower levels observed in the follicular phase of menstruation or while using contraceptive medication compared with males (16). Changes in the free cortisol response could lead to alterations in sIgA concentration as cortisol plays a role in the movement and functional capabilities of several immune cells including B cells (13). It has been reported (16) that gender-related secretion rates may be attenuated when male and females of the same age group are compared. The results of the present study exhibit similar results for males and females, with both genders showing non-significant increases in post-event means compared with pre-event means. Additionally both genders exhibit non-significant increases in sIgA concentrations 17 h post-event however only males were observed to have a signifanct increase in sIgA concentrations 61 h post event. It is possible that male athletes returned to regular training follwing the event prior to female athletes and therefore the elevated sIgA concentrations observed at 61 h post-event for males could be attributed to increased neural activation associated with the resumption of high intensity training. Additionally, it is possible that differences in hormonal release from varying phases of the menstrual cycle or the use of oral contraceptive medication could have reduced the sIgA concentration in females,

however as no information was gathered regarding which phase of the menstrual cycle each female particpant was in at the time of sample colelction, this remainds anecdotal.

The present study observed absolute sIgA concentration in elite SLS athletes pre and post- competition, which is consistent with applied research methodologies that have investigated the impact of training on sIgA concentration and the relationship between sIgA concentration and competitive performance in swimmers leading into National Championships and the 1998 Commonwealth Games (11, 35). Salivary IgA has been identified to be subjected to more short-term variations compared with other salivary proteins (6, 36) in addition to greater within- and between-subject variations in athletes such as elite rowers and swimmers (27). Variation in the number of subjects, the demands of exercise, saliva collection and analysis methods reduces the ability for direct comparison between the results of the present study and others (9, 11, 35). As a result of the high variability of sIgA, studies with small sample sizes, such as the present study, may be less likely to detect real changes in salivary proteins and therefore any results should be interpreted with caution (15).

The present study found a significant increase ($p < 0.05$) in sIgA concentration 61 h post-event in males, compared with all other time-points. In addition, non-significant increases in sIgA concentrations were observed post-event when compared to pre-event in both genders, which suggests increased sympathetic nervous system activation associated with high intensity and prolonged exercise could have led to an increased secretion of sIgA into the buccal cavity. However, due to the applied population and resultant limited sample size available for participation in the present study as well as the high within- and between-subject variations in sIgA it is difficult to draw unequivocal conclusions from the results of the present study. It is possible that a cumulative effect of substantial physical conditioning over several years has had an adverse effect on the mucosal immune system, resulting in abnormal variations in sIgA concentration. Further longitudinal research with a greater sample size using the IPRO device should be conducted to determine the immunological response to elite SLS Ironmen and Ironwomen competition and the validity and reliability of the device for use in an applied sport setting.

PRACTICAL APPLICATIONS

This study demonstrated the atypical response of elite surf lifesaving athletes to that previously reported for athletes of similar disciplines such as swimming and kayaking, highlighting the variability of immunosuppressive responses. The use of a point of care, field-based saliva analysis system may provide coaches and athletes with the ability to individually assess the immediate effects of training and competition on the immune system. Swift feedback on immune functioning following physical exertion could allow coaches and athletes to monitor immunosuppression, predict the risk of infection and adjust training periodization and recovery sessions accordingly. Elite SLS athletes and coaches may need to implement stringent precautions while tapering prior to major competitions, to reduce the risk of upper respiratory tract infection as a result of lowered sIgA concentrations in response to reduced training load. The results of the present study indicate the sIgA response in the days following SLS competition may vary between genders and therefore individualised recovery protocols should be implemented to minimise the risk of acquiring respiratory infections post-exercise and reduce the risk of resuming regular training prematurely.

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